TOWSON UNIVERSITY
OFFICE OF GRADUATE STUDIES

EFFICACY OF ANTIVIRAL DRUG COMBINATIONS AGAINST EQUINE HERPES VIRUS TYPE 1 *IN VITRO*

by

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A thesis

Presented to the faculty of

Towson University

in partial fulfillment

of the requirements for the degree

Master of Science

Department of Biological Sciences

Towson University
Towson, Maryland 21252

August, 2015

TOWSON UNIVERSITY
COLLEGE OF GRADUATE STUDIES AND RESEARCH

THESIS APPROVAL PAGE

This is to certify that the thesis prepared by Midrelle Nandjou entitled "efficacy of antiviral drug combinations against Equine Herpes Virus Type 1 *in vitro* ", has been approved by her committee as satisfactory completion of the requirement for the degree of Master of Science.

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ACKNOWLEDGEMENTS

I would first like to thank my principal investigator, Dr. Barry Margulies, for the support, training, and teaching he provided me during my time at Towson University and in TUHVL. He really helped me in developing the direction of this project, giving me words of encouragement, and support. In addition, I would like to thank the other members of my committee Drs. Renee Dickie and Matthew Hemm. They both provided encouragement, suggestions, and support leading to the successful completion of this project. I would also like to thank the previous and current lab members of the TUHVL who have taught me and assisted me over the past two years: Sarah Fargis, Brian Green, Thammy Etienne, Amandeep Samra, Sogand Fartash-Naini, Kevon Sampson, Jenna Hickey, Benjamin Harding, Brian White, Ransel Palatino, Jackie Hauffman, Kaitlyn Biles, and Damian Reinhart. I also thank my family, and friends who have supported and motivated me through this journey. I am also thankful to Dr. Balasuriya from University of Kentucky who provided the virus used in this study. Finally, I would like to thank the NIH for their R15 award and the Towson University Graduate Student Association for financially supporting this research.

ABSTRACT

Efficacy of antiviral drug combinations against equine herpes virus type-1 *in vitro*

Midrelle Nandjou

Equine herpes virus type-1 (EHV-1) is responsible for a neurological infection in horses called equine herpes myeloencephalopathy (EHM). There is currently no specific treatment or vaccine licensed against the neuropathogenic EHV-1 strain (T953). In this study, the effect of combining the antiviral drugs cidofovir, foscarnet, and acyclovir was investigated. Specifically, we looked at the effect of using cidofovir + foscarnet and cidofovir + acyclovir against neuropathogenic EHV-1 (T953) propagated in equine dermal fibroblast (E. Derm) cells. This is the first study to look at the effect of combining different antiviral drugs against EHV-1 (T953). The combination of cidofovir (4 µg/mL) + acyclovir (10 µg/mL) and cidofovir (7.5 µg/mL) + foscarnet (5 µg/mL) were found to be non-toxic and to have an additive and no effect against EHV-1 (T953), respectively. These drug concentrations were also found to be non-toxic to E. Derm cells.

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CHAPTER I:

INTRODUCTION

Herpesviruses are a family of viruses which infect hosts ranging from vertebrates to invertebrates. Herpes comes from the Greek word “herpein”, meaning to creep, which characterizes the chronic and latent infections caused by herpesviruses. All herpesviruses are enveloped and possess a double-stranded linear genome with an icosahedral capsid (Strauss, 2002). The capsid is surrounded by a tegument which is made up of viral proteins (Whitley, 2001; Strauss, 2002). Herpesviruses infecting mammals are divided into three subfamilies, *alphaherpesvirinae*, *betaherpesvirinae*, and *gammaherpesvirinae*, based on their tropism, tissue culture behavior and pathogenicity (McGeoch *et al*., 2000; Davison, 2002).

Three herpes virus strains from the *Alphaherpesvirinae* subfamily infect horses: equine herpes virus type-3 (EHV-3), which causes superficial pock-like ulcers on the horse external genital organs (Blanchard *et al*., 1992); equine herpes virus type-4 (EHV-4), which causes respiratory distress (Borchers *et al*., 1999); and equine herpes virus type-1 (EHV-1) which is similar in structure to EHV-4 and also causes respiratory distress (Patel and Heldens, 2005). However, although both EHV-4 and EHV-1 may cause respiratory distress, EHV-1 is the main cause of abortions, paresis and neonatal foal deaths (Patel and Heldens, 2005). A particularly serious aspect of EHV-1 infection is that pregnant mares abort their fetuses. Furthermore, severe neurological signs may be observed in these infected horses (Carrol and Westbury, 1985).

Equine herpes virus type-1 (EHV-1) is a large enveloped virus. It is a member of the *Alphaherpesvirinae* subfamily and part of the *Varicellovirus* genus. EHV-1 has a 150-

kbp double-stranded DNA genome consisting of 80 open reading frames (ORFs) (Burton *et al*., 2001). The viral genome is divided into a unique short (U_S) and unique long (U_L) region, which are both flanked by inverted internal (IR_S and IR_L, respectively) and terminal repeat sequence (TR_S and TR_L, respectively) (Crabb and Studdert, 1996; Telford *et al*., 1992). Most of the ORFs (76 of them) encode unique genes while the rest are duplicated in the TR_S (Telford *et al*., 1992). The EHV-1 genome is enclosed in an icosahedral nucleocapsid similar to other members of the *Alphaherpesvirinae* subfamily (Turniten *et al*., 1981). Also, the shedding of EHV-1 upon administration of corticosteroid or a variety of noxious stimuli suggests that the virus establishes latency in the host in a manner similar to that of herpes simplex virus (HSV) or varicella-zoster virus (VZV) in man (Edington *et al*., 1985).

EHV-1 is a ubiquitous respiratory viral pathogen causing serious economic losses in the horse industry worldwide (Allen and Bryans, 1986). EHV-1 infection usually occurs via inhalation of the infectious virus or contact with nasal discharges from infected horses (Patel *et al*., 1982). The virus may also be transmitted by contact with aborted fetuses or placental fluids or placentas from infected horses (Dunowska, 2014). The mucosa of the upper respiratory tract is the primary site for EHV-1 replication (Van Maanen, 2002). The virus then disseminates via a leukocyte-associated viremia, which enables EHV-1 to spread to end-vessel endothelial cells in the uterus and central nervous system (Allen and Bryans, 1986). In the uterus and central nervous system, virus replication may result in vasculitis and perivasculitis, thus leading to abortion and myeloencephalopathy, respectively (Vanderkerckhove *et al*., 2010).

EHV-1 was shown to enter cells in vitro via a number of different pathways, either by direct fusion with an endosomal membrane or via endocytosis followed by fusion with an endosomal membrane (Azab *et al*., 2013). The virus has been found to use equine major histocompatibility complex 1 (MHC-1) (Kurtz *et al*., 2010) and cellular integrins (Azab *et al*., 2013) as receptors to gain entry into cells. There are also additional yet unidentified receptors used by the virus for entry into some cell types (Azab and Osterrieder, 2012). However, it is currently unknown what factors determine the method of entry of EHV-1 into cells, and whether EHV-1 mechanisms of entry are the same *in vivo* as those described *in vitro*.

Initially, EHV-1 infects epithelial cells of the nasal mucosa or nasopharynx, resulting in epithelial cell damage (Gryspeerdt *et al*., 2010). The respiratory epithelium usually starts to recover from the virus-induced damage 3-5 days post infection (Gryspeerdt *et al*., 2010). However, unlike the situation observed with other alphaherpesviruses (Glorieux *et al*., 2011), EHV-1 virions do not infect the basal membrane *in vivo* (Gryspeerdt *et al*., 2010) or *in vitro* in nasal explants (Vandekerchkhove *et al*., 2010). Even though, the basal membrane is intact, individual EHV-1 infected cells, comprise predominantly of monocytes and T-lymphocytes, can be observed in the connective and lymphoid tissues of the respiratory tract within 24-48 hours following experimental infection with the virus (Gryspeerdt *et al*., 2010). Therefore, the ability of EHV-1 to infect cells of the immune system may enable it to cross the basal membrane and disseminate to other organs within the body, such as the pregnant uterus and the central nervous system (CNS) (Dunowska, 2014).

Infection of the respiratory epithelial cells leads to pneumonia or respiratory distress and the virus is subsequently drained into the local lymphoid tissue, including the submandibular, retropharyngeal, and bronchial lymph nodes (Harless and Pusteria, 2006). EHV-1 envelope glycoprotein K (gK) appears to mediate entry into these respiratory epithelial cells, viral replication, and cell-to-cell viremia (Neubauer and Osterrieder, 2004). Also, destruction of the respiratory tract epithelial cells and lymphoid tissue (secondary to viral replication and budding) results in local lymphadenopathy (Harless and Pusteria, 2006).

EHV-1 infected leukocytes disseminate the virus to various organs such as the CNS and the uterus. Infection of endothelial cells of the blood vessels in the gravid uterus causes severe vasculitis and multifocal thrombosis, which are believed to be responsible for abortion (Smith and Borchers, 2001a). The primary factors responsible for the initiation of endothelial cell infection from EHV-1 positive leukocytes are still unclear. However, it is hypothesized that the process may be facilitated by changes in the cell surface molecules expressed by the endothelial cells of the gravid uterus during late stages of pregnancy (Smith *et al*., 2001b).

Infection of endothelial cells of the blood vessels in the CNS leads to severe vasculitis and thrombosis, which are believed to be responsible for a neurological infection in horses (Edington *et al*., 1985). This neurological infection leads to reactive equine herpes myeloencephalopathy (Greenwood and Simson, 1980), with symptoms ranging from mild ataxia to complete paraplegia requiring euthanasia of affected animals (Wilson, 1997). The efficiency with which the virus can cross the basal membrane (via infected cells of the immune system) may be related to its virulence, as the number of

EHV-1 infected cells below the basal membrane appears to be higher in ponies infected with the neurovirulent strain of EHV-1 as compared to those infected with the non-neurovirulent strain (Gryspeerdt *et al*., 2010). Other as yet as unidentified mechanisms may play a role in the translocation of EHV-1 to endothelial cells of the CNS. It is also possible that there are physiological triggers acting at the level of EHV-1-infected leukocytes to initiate reactivation of the virus (Smith *et al*, 2001b). Nevertheless, the ability to infect endothelial cells is an important biological feature of the virus since highly virulent EHV-1 isolates were found to be more endotheliotropic than those with low virulence (Smith *et al*., 2000).

Shattering outbreaks of equine herpes myeloencephalopathy (EHM) caused by variants of EHV-1 have being reported with increasing rates throughout North America and Europe (Perkins *et al*., 2009). This resulted in the US Department of Agriculture's Animal and Plant Inspection Service (USDA- APHIS) to classify EHM as a potentially emerging infectious disease (USDA APHIS, 2007). In the United States, the associated case-fatality rate of EHM ranged from 20% in some states to as high as 50% in others (Slater *et al*., 2004). Moreover, in 2005 significant outbreaks of EHM were reported in Canada, South Africa, Switzerland, Ireland, and other European nations (Goehring *et al*., 2006). These outbreaks were mostly common among stables and mature horses, suggesting that age and management conditions (confined vs pastured) may comprise risk factors for the development of EHM (Kydd *et al*., 2012).

Within open reading frame 30 (ORF30), encoding the viral DNA polymerase, a single nucleotide substitution is strongly associated with a change in pathology from respiratory signs or abortions to neurological signs (Nugent *et al*., 2006). The exchange

of adenine for guanine at position 2254 (ORF30; A2254 - G2254) results in an asparagine (N) to aspartate (D) substitution at amino acid position 752 (N752 - D752) (Nugent *et al*., 2006). This genotype has been reported as the causative agent of 30 out of 32 investigated outbreaks of EHM occurring in the United Kingdom and the United States between 2001 and 2006 (Allen, 2007). Moreover, the ability of EHV-1 strains having G2254 to induce neurological signs has also been proved through experimental infection of horses (Goodman *et al*., 2007). It should also be noted that all strains of EHV-1 may be able to induce neurological symptoms since, 14% to 24% of EHV-1 isolates from horses with EHM do not have the neurological marker (G2254) (Pusteria *et al*., 2008). Neuropathogenic EHV-1 strains (D752) are also able to replicate *in vivo* more efficiently and reach 10-fold higher levels of leukocyte-associated viremia than what is observed in horses infected with non-neuropathogenic EHV-1 (Van de Walle *et al*., 2009).

The relationship between all the EHV-1 strains and their equine hosts are complex and not fully understood since it is currently unclear which viral and host factors are important for the clinical outcome of EHV-1 infection or EHM in an individual animal (Dunowska, 2014). Also, not all EHV-1 isolates with N752 to D752 substitution can induce a neurological infection, and not all cases of EHM are caused by the D752 mutant (Cuxson *et al*., 2014). Thus, it is likely that the viral markers of neurovirulence are more complex than this single amino-acid substitution (Pronost *et al*., 2010). Henceforth, the importance of N752 to D752 substitution should not be over-interpreted.

Primary infection with EHV-1 usually results in the establishment of latency (Welch *et al*., 1992). Latently infected animals harbor EHV-1 in the episomal form in the trigeminal ganglia (Slater *et al*., 1994) or in lymphoid cells (Chester *et al*., 1997). Via

extrapolation of data from other alphaherpesviruses, EHV-1 most likely infects sensory nerve endings in the nasal cavity, then travels by retrograde axonal transport to the trigeminal ganglia where it becomes latent (Zaichick *et al*., 2011). However, the latent virus does not synthesize any viral membrane glycoproteins, and infected cells are therefore not recognized by the immune system (van der Meulen *et al*. 2006). Also, even though latently infected horses are asymptomatic, they are still able to shed the virus, a process called asymptomatic shedding (Allen, 2006).

Under some conditions, the latent virus can reactivate from latency, a process called recrudescence (Dunowska, 2014). But the triggers for EHV-1 recrudescence, as well as the molecular mechanisms underlying this process, are poorly understood. Nonetheless, the virus can be reactivated in real life via stressful conditions such as transport, sales, competitions or unsettled social structure (Dunowska, 2014). Reactivation by stress stimuli can also be observed in other human alphaherpesviruses (e.g. herpes simplex viruses), whereby the stress stimuli act on the neuron or at a peripheral site innervated by the infected ganglion (Preston and Efstathiou, 2007). Also, physiological immunosuppression such as that observed during pregnancy in mares may cause reactivations of EHV-1 (Noronha and Antczak, 2012). It should be noted that recrudescence of latent EHV-1 may (Gibson *et al*., 1992) or may not (Edington *et al*., 1985) result in clinical disease. However, following EHV-1 recrudescence, the latently infected horse becomes infectious and a potential source of EHV-1 transmission to susceptible animals. The source of EHM infection is thought to be EHV-1 that has reactivated locally within the blood vessels and possibly, by extrapolation to the CNS (Slater, 2007). This local reactivation can occur with or without concurrent lytic

respiratory infection and hence, with or without shedding of the virus in nasal secretions (Slater, 2007). Thus, the initial respiratory infection that led to the establishment of latency could have happened possibly months to years before an EHV-1 abortion or neurological infection (Allen, 2006).

Outbreaks of EHM are thought to be initiated by viral reactivation and nasal shedding of neuropathogenic (D752) or non-neuropathogenic strains (N752) of EHV-1 by stressed latently infected carriers (Allen and Timoney, 2007). Almost all horses have been infected with a strain of EHV-1 but some of them do not develop EHM (Pusteria *et al*., 2008). It is currently unknown what causes some of the horses to develop the deadly neurological infection. Thus, prevention is usually difficult since many horses are latently infected, allowing the virus to circulate silently in horse populations (Patel and Heldens, 2005). The latently infected horse is usually asymptomatic, although mares harboring latent EHV-1 strains can abort their foals (Allen *et al*., 2004). Even though the primary site of latency is the lymph nodes associated with the respiratory tract, latent virus has also been found in circulating lymphocytes and sensory nerve-cell bodies of the trigeminal ganglia (Chesters *et al*., 1997). Natural immunity to infection by EHV-1 appears to be incomplete or short-lived, and reinfection with the same or related strains together with production of clinical signs have been documented (Bryans, 1969).

Currently, there are two inactivated, high antigen load vaccines licensed in North America for the prevention of both respiratory and abortive disease induced by EHV-1: Pneumabort-K® (Pfizer Animal Health, New York, NY) and Prodigy™ (Intervet Schering-Plough Animal Health, Kenilworth, NJ) (Goehring *et al*., 2010). There is also a modified live vaccine (MLV) called Rhinomune® (Boehringer Ingelheim Vetmedica,

Inc., St. Joseph, MO), which is used in North America for the prevention of only the respiratory disease (Goehring *et al*., 2010). Although these vaccines are used to prevent EHV-1 infections (respiratory and abortive disease), they are not fully protective (Kydd *et al*., 2006; Goodman *et al*., 2006) against the virus. This is because, outbreaks of both respiratory and abortive disease have been reported in some vaccinated horses (Buchner and Mostl, 1998). In general, it is advisable to immunize foals older than 3-5 months, followed by a second immunization within 4 to 6 weeks (Paillot *et al*., 2008). Depending on the type of vaccine, the immune system also needs to be boosted by a single vaccination every 3 or 6 months (Paillot *et al*., 2008). Additionally, to prevent EHV-1-induced abortions, pregnant mares are usually vaccinated at the 5th, 7th and 9th months of pregnancy (Paillot *et al*., 2008). However, these vaccines (Pneumabort-K®, Prodigy™ and Rhinomune®) do not reliably block the respiratory or abortive infection, the development of viremia, or the establishment of latency. This is because cases of EHM have been observed in horses regularly vaccinated against EHV-1 at 3-5montlhly intervals with the modified live vaccines (MLV) (Friday *et al*., 2000; Henninger *et al*., 2007). Despite the widespread use of the aforementioned vaccines, there is still no vaccine licensed against the EHV-1 strain responsible for causing EHM in horses (Patel and Heldens, 2005).

Some antiviral drugs have been tested *in vitro* for their efficacy in inhibiting EHV-1 replication (Gibson *et al*., 1992; Boyd *et al*., 1987; de la Fuente *et al*., 1992; Rollinson and White, 1983). However, the *in vivo* efficacy of these antiviral drugs against the mutant EHV-1 strain (D752) responsible for EHM is still unclear. In 2007, Garre *et al*. conducted an *in vitro* efficacy study of acyclovir, ganciclovir, cidofovir, foscarnet,

adefovir and the acyclic nucleotide analog PMEDAP (9-[2-(phosphonomethoxy) ethyl]-2, 6-diaminopurine) against different isolates of EHV-1 (abortigenic and neuropathogenic) (Garre *et al*., 2007). In their *in vitro* efficacy study, Garre *et al*. found ganciclovir to be the most potent antiviral compound against all the EHV-1 isolates, while cidofovir had a strong effect on plaque size. However, there is still no specific treatment licensed against the neuropathogenic (D752) strain of EHV-1 since treating the virus and infection is very challenging (Pusteria *et al*, 2009). Because of the lack of a specific treatment against neuropathogenic (D752) EHV-1, the affected horses are managed via supportive nursing, nutritional care, and reduction of CNS inflammation (Pusteria *et al*, 2009). Moreover, isolation of affected horses, segregation and monitoring of exposed horses, quarantine measures, and euthanization of affected horses are used to prevent the spread of the virus.

In the study presented here, we explored a potential antiviral treatment regimen specific for a neuropathogenic isolate of EHV-1 (T953 isolate). Since numerous previous studies reported *in vitro* efficacy of antiviral drugs when used alone (Gibson *et al*., 1992; Boyd et *al*., 1987; de la Fuente *et al*, 1992; Rollinson and white, 1983; Garre *et al*., 2007), our study investigated the effects of using these antiviral drugs in combination, especially because their molecular targets are different. Previous studies reported synergistic inhibition of human cytomegalovirus replication *in vitro* by ganciclovir and foscarnet (Manischewitz et al., 1990). Henceforth, the aim of the present study was to determine and compare any possible synergistic effect between acyclovir (ACV), foscarnet (FOS), and cidofovir (CDV) against neuropathogenic EHV-1 (T953 isolate) via an *in vitro* efficacy study. Specifically, we focused on the therapeutic effects of the

following drug combinations: cidofovir + foscarnet and cidofovir + acyclovir. Also, the aforementioned drugs (acyclovir, foscarnet and cidofovir) were chosen because of their ability to penetrate the CNS (Razonable, 2011).

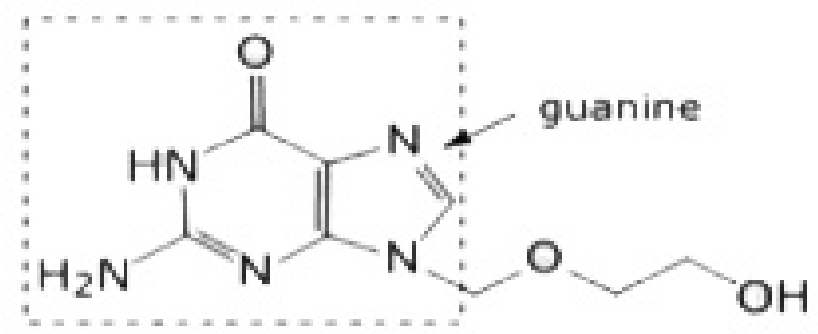
Acyclovir (Figure 1) is a synthetic nucleoside analog of deoxyguanosine that inhibits viral DNA synthesis through competitive incorporation during viral DNA synthesis, thereby leading to viral DNA chain termination (Figure 1) (Razonable, 2011). Nucleoside analogs are transported into cells and then phosphorylated by the viral thymidine kinase, produced during a lytic infection (Fyfe *et al*., 1978; Furman *et al*., 1984). Later, cellular kinases add two more phosphate groups (Figure 1). At this point, the nucleoside analog has three phosphate groups attached to its apparent 5' end. The viral DNA polymerase then incorporates this triphosphorylated nucleoside analog into the growing viral DNA chain. Due to the lack of a free 3' hydroxyl group, incorporation of the nucleoside-triphosphate analog usually terminates viral DNA chain synthesis. Cidofovir (Figure 2) is a nucleotide analog which is already phosphorylated as if it were a monophosphorylated product. As with ACV, cellular kinases triphosphorylate the drug creating a competitive inhibitor of viral DNA polymerase, thereby halting viral DNA synthesis (Lea and Bryson, 1996). Foscarnet is a nonnucleoside pyrophosphate analog which selectively inhibits pyrophosphate binding to viral DNA polymerases, thus suppressing viral replication (Balfour *et al*., 1996).

It should be noted that both nucleoside (ACV) and nucleotide (CDV) analogs are very efficient at terminating viral DNA synthesis without affecting the host cell DNA synthesis. Firstly, the viral thymidine kinase is the only enzyme capable of adding the first phosphate group to nucleoside analogs, whereas the cellular ortholog cannot.

Therefore, nucleoside analogs are only activated in infected cells. Also, the viral DNA polymerase has a higher affinity for the given nucleoside or nucleotide triphosphate analogs, relative to the drug's affinity for host DNA polymerase. This allows both acyclovir and cidofovir to be effective against most herpes infections and yet safe for the host. In the case of foscarnet which is a pyrophosphate analog, the viral DNA polymerase has a higher affinity for foscarnet triphosphate allowing foscarnet to be effective against most herpes infections and yet exhibit some selective toxicity.

Our studies look specifically at the antiviral drug combinations (CDV+ FOS, CDV + ACV). The former drug combination of CDV and FOS was used because each of the drugs acts on a unique site of the viral DNA polymerase. The latter combination of CDV and ACV was tested because, when used alone, both CDV and ACV were found to have some protective effect against the EHV-1 isolate (T953) used in this study. A possible synergistic effect among the drug combinations may lead to a novel treatment regimen specific against neuropathogenic EHV-1 (T953) and this may also delay or prevent the occurrence of drug resistant neuropathogenic EHV-1 (T953) strains. The safety and efficacy of these drug combinations were tested *in vitro* (on equine dermal fibroblast cells) via cytotoxicity and plaque assays on equine dermal fibroblast cells, respectively.

A.



B.

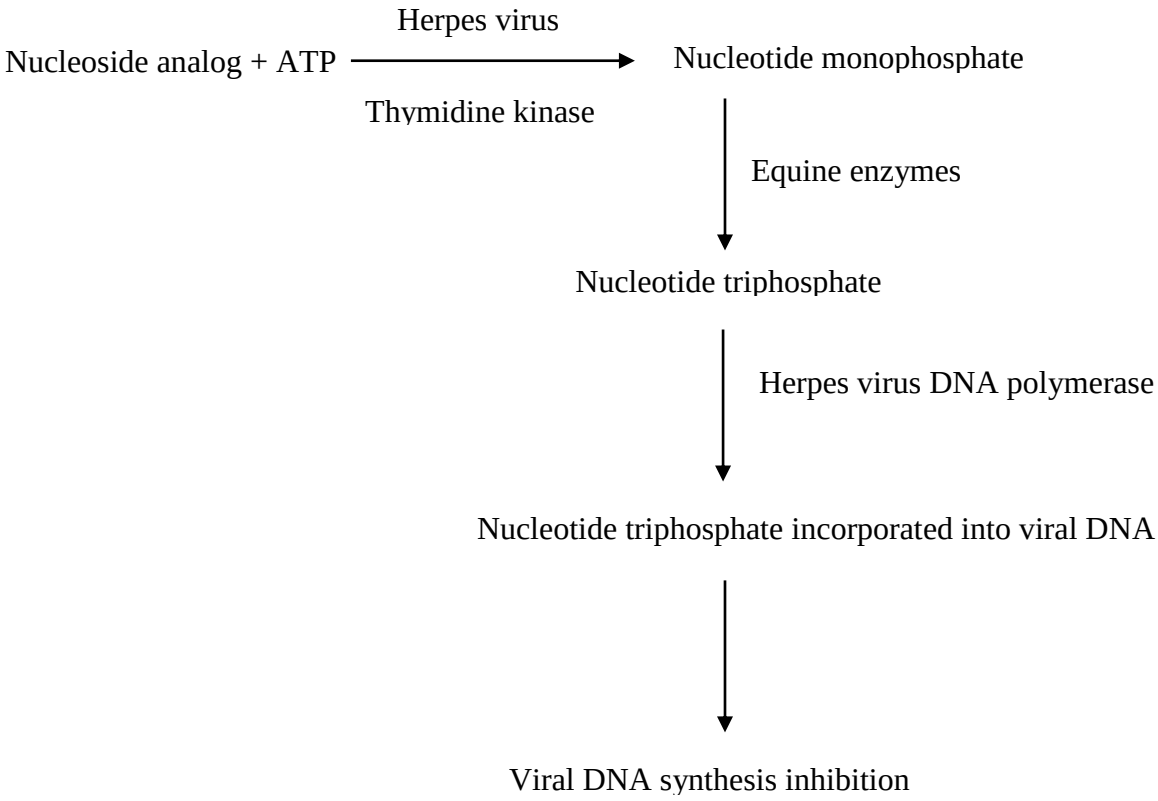
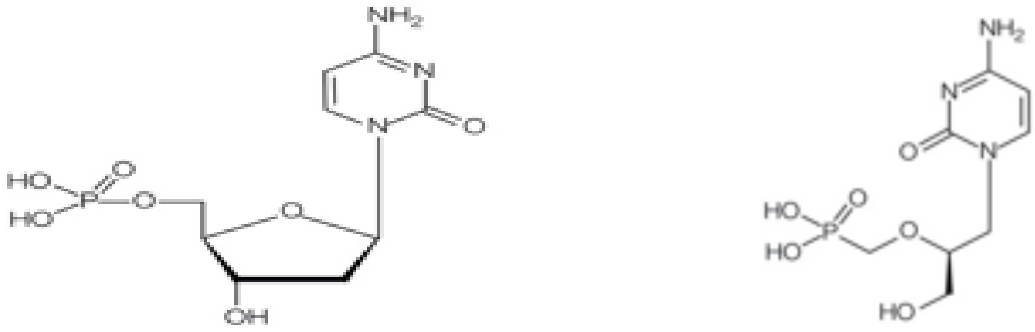


Figure 1: A. Chemical structure of acyclovir. B. Pathway of phosphorylation of nucleoside analog by viral thymidine kinase and cellular kinases.

A.



Natural nucleotide (2'-deoxycytosine 5'-monophosphate) Cidofovir

B.

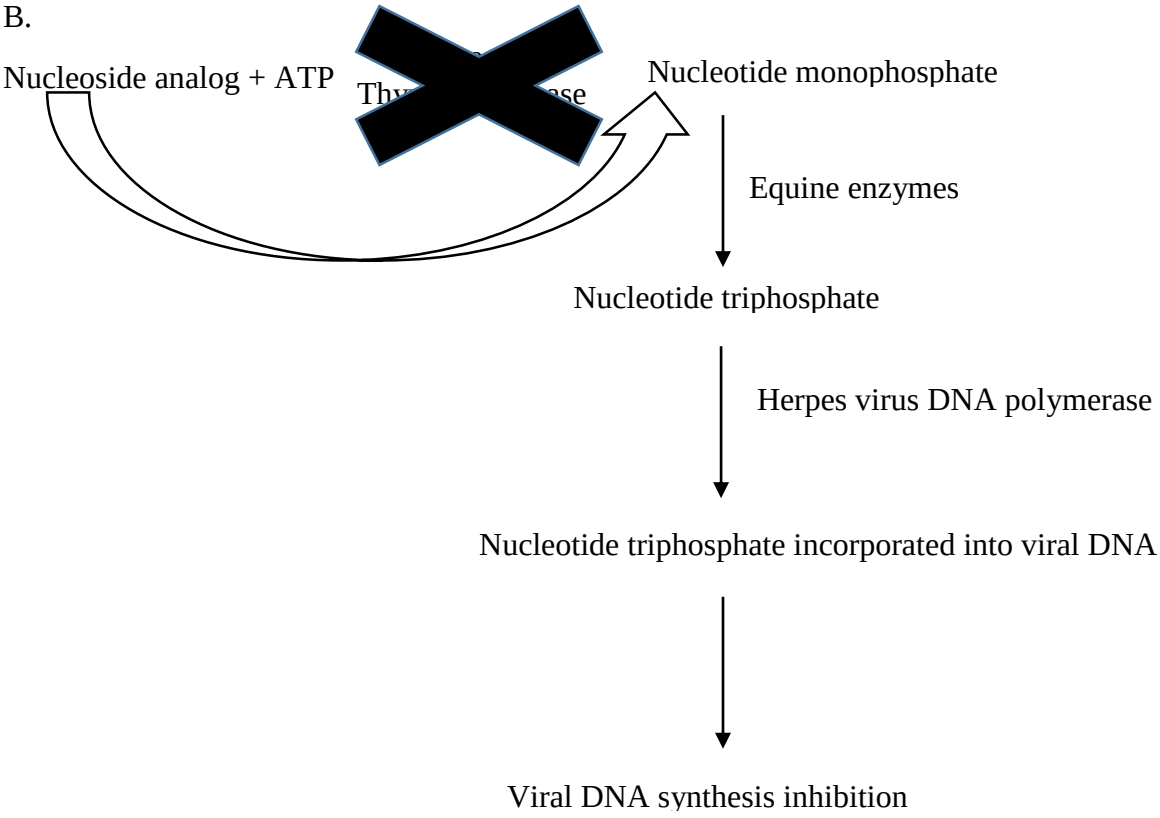


Figure 2: A. Chemical structure of a natural nucleotide and cidofovir. B. Pathway of phosphorylation of nucleotide analog by cellular kinases.

Chapter II:

MATERIALS AND METHODS

Cells and Virus

Equine dermal fibroblast (E. Derm) cells (ATCC #CCL-57) were maintained in Minimum Eagle Medium (MEM) (Mediatech Inc., Manassas, VA) supplemented with 10% fetal bovine serum (Hyclone), 1% glutamax (Gibco, Carlsbad, CA) and 1% antibiotic/antimycotic (Hyclone, Logan, UT). Cells were cultured in T-75 polysterene cell culture flasks (BD Falcon, Franklin Lakes, NJ) in 25mL of complete MEM, as described above. The cells were grown in a 37 $^{\circ}$C incubator with 5% CO_2.

Equine herpes virus-1 (EHV-1) T953 isolate (with the neurological marker D752) strain (gift of Dr. Balasuriya, University of Kentucky) was used in the cell culture study. The virus was grown and titered (see Plaque Assay) and stored at -80 $^{\circ}$C.

Antiviral Drugs

Acyclovir (Advanced Scientific, Ft. Lauderdale, FL), cidofovir (Abmole, Harbour City, Kowloon Hong Kong), and foscarnet (Sigma-Aldrich, St. Louis, MO) were used for this study. ACV at 5 mg/mL was solubilized in DMSO (Sigma-Aldrich, St. Louis, MO) then in DPBS (Hyclone) to working concentrations. FOS at 5mg/mL was solubilized in distilled water then in DPBS (Hyclone) to working concentrations. CDV at 5 mg/mL was solubilized in distilled water then the pH was adjusted to six using NaOH.

Cytotoxicity Assay (MTT Assay)

A cytotoxicity assay was used to determine the cytotoxic concentrations of the drugs when used alone or in combination. Antiviral drug concentrations of 1, 5, 10, 15, 20, 25, and 30 µg/mL were used to determine the cytotoxicity concentration at 50% (CC_{50}) of the antiviral drugs when used alone. For the drug combinations (acyclovir + cidofovir and foscarnet + cidofovir), drug concentrations far below the CC_{50} of each antiviral drug (See Results) were used for the MTT assay in order to confirm the low cytotoxicity levels of these chosen concentrations.

For this study, two different MTT assay protocols were used. In the first protocol, a 96-well culture plate (BD Falcon, Franklin Lakes, NJ) was seeded with 5×10^3 E. Derm cells per well, in 100 µL of MEM lacking phenol red (Mediatech Inc). The cells were grown overnight in a 37 °C incubator with 5% CO_2. Twenty-four hours later, different concentrations of antiviral drugs (alone or in combination) were added to the wells. Untreated control wells received DPBS (Hyclone) instead of drug. Background control wells received MEM only (with neither E. Derm cells nor antiviral drug). After 24 h, 10 µL of 1mg/ml MTT reagent (Abnova, Taipei City, Taiwan) was added to each well and incubated for 4 hours at 37 °C with 5% CO_2. After 4 hours, formazan crystals were formed at the bottom of the wells. The content in each well was discarded and 100 µL of crystal dissolving reagent (Abnova) was added to each well to dissolve the formazan crystals. Finally, absorbance was measured using a spectrophotometer at 570nm, and cell viability was calculated using the following formula:

Percentage of viable cells = (Odt – ODd/ ODc – ODd) x 100%

where Odt was the absorbance from cells incubated with antiviral compounds, ODd was the absorbance from the background control and ODc was the absorbance from the untreated cells.

For the combination of ACV + CDV, a different approach was used for the MTT assay. Briefly, a 96-well culture plate (BD Falcon, Franklin Lakes, NJ) was seeded with 1 x 10^4 E. Derm cells per well as above. Cells were incubated for 48 h with different drug concentrations (or DPBS) in a 37 $^{\circ}$C incubator with 5% CO_2. Two days after the addition of drug, the content in each well was removed and the wells were rinsed with 100 µL DPBS. Background control wells received MEM only (neither E. Derm cells nor antiviral drug). Then, 10 µL of 5 mg/mL MTT reagent (Sigma-Aldrich) was added to each well and incubated for two hours at 37 ^{o}C with 5% CO_2. After four hours post incubation, the content in each well was discarded and 200 µL of DMSO (Sigma-Aldrich, St. Louis, MO) was added to each well. Finally, absorbance was measured using a spectrophotometer at 570nm, and cell viability was calculated using the formula mentioned above.

Cell Culture Study

A cell culture study was used to determine the effective drug concentration when used alone or in combination. A 12-well culture plate (BD Falcon) was seeded with 9.12 x 10^5 E. Derm cells per well, in 2 mL of MEM per well. The cells were allowed to grow in a 37 $^{\circ}$C incubator with 5% CO_2. Twenty-four hours later, 2 mL of media from each well was collected, and stored at -80 $^{\circ}$C for later plaque assay analysis. Two mL of MEM plus different drug concentrations were then added to each well. Control wells did not

receive any drug. The next day, the medium was collected, stored and fresh media plus drug was added as above. Wells were either infected with EHV-1 (T953) at an MOI of 0.1 or left uninfected. Up until 7 days post-infection (d.p.i.), the medium was collected, stored and changed. At 4 d.p.i., the cells were observed with an Accu-Scope 3032 microscope and images were captured using a Sony Cybershot DSC-H2 12x optical zoom digital camera. Multiple shots of each well were taken at different focal planes and combined together using Zerene Stacker software (Zerene Systems, Richland, WA).

Plaque Assay

A plaque assay was used to determine viral titers of the samples collected from the cell culture study. First a 0. 75% methylcellulose overlay was prepared by boiling 150 mL of DPBS and adding 3.75 g of methylcellulose powder (Sigma-Aldrich). The resulting mixture was autoclaved and chilled on ice. Once the suspension was cleared, 350 mL of MEM, as mentioned above, was added to the PBS/methylcellulose mixture, and the methylcellulose overlay was stored at 4 $^{\circ}$C.

To conduct the assay, a 6-well cell culture plate (BD Falcon) was seeded with 3.6 x 10^6 E. Derm cells per tray, in 4 mL of MEM per well. Twenty-four hours later, the medium was removed from each well and 0.2mL of samples containing virus or not were added to each well. As needed, some samples were serially diluted. The sample in the wells were placed at 37 $^{\circ}$C in a CO_2 incubator for two hours during the adsorption period. Every 10mins during the adsorption period, the tray was moved forward and backward to evenly distribute the virus or sample. After two hours of adsorption, 4 mL of the 0.75% methylcellulose overlay was added to each well and the tray was incubated at 37 $^{\circ}$C/5% CO_2 for four days. The methylcellulose was discarded and 1 mL of 1% crystal violet

(Sigma-Aldrich) in 50% ethanol (Pharmco-AAPER, Brookfield, CT) was added to each well. After 30 mins, the crystal violet stain was washed off using tap water. Trays were set out to dry and the number of plaques were counted. Countable wells contained between 30-300 plaques. The number of counted plaques were used to determine the effect of drug concentrations on the virus using the following formula:

Drug Effect = (1 – (plaque number/number of cells seeded in each well))

The viral plaque counts were also used to determine the viral titer of infected wells pre-treated with antiviral drugs (alone or in combination) using the following formula

Viral titer = number of plaques X dilution factor/ volume of inoculum (0.2mL)

Statistical Analysis

The effects of the drug combinations were assessed using the combination index (CI) method as described elsewhere (Zhao *et al*., 2004) and these effects were used to generate CI values using Compusyn software (Chou and Talalay, 1984) for Windows.

Chapter III:

RESULTS

Cytotoxicity assay

The toxic effect of antiviral drugs on E. Derm cells was measured via an MTT assay. For all the antiviral drugs used alone (cidofovir, acyclovir, and foscarnet), concentrations of 1, 5, 10, 15, 25, and 30 µg/mL were tested. The CC_{50} (the concentration at which 50% of the cells exhibited cytotoxicity) of each individual antiviral drug was then derived via its respective graph. ACV was found to have a CC_{50} value around 23 µg/mL (Figure 3); CDV's CC_{50} value was around 27.5 µg/mL (Figure 4), while that of FOS was around 10 µg/mL (Figure 5).

Once the CC_{50s} of the individual drugs were determined, concentrations far below their respective CC_{50} were chosen for their combinations in order to mitigate any potential toxic effects of their use. Although these chosen drug concentrations were far below the CC_{50} of their respective individual drugs, their toxic effects when combined together was still tested via the MTT assay. This was done in order to confirm the safety of our chosen concentrations for the drug combinations.

For the combination of CDV plus FOS , the toxicity of FOS at 5 µg/mL combined together with CDV at 1, 5, 12.5, and 17.5 µg/mL was chosen to be tested. It should be noted that these chosen concentrations were far below their respective individual CC_{50}. CDV combined with FOS at these chosen concentrations were found to be non-toxic for the cells since the lowest observed cell viability was around 85% (Figure 6).

The toxic effect of ACV at 10 µg/mL combined with CDV at 1, 2.5, 5, 7.5, and 10 µg/mL each were also chosen to be tested. These concentrations were also far below their respective individual CC_{50}. The combination of CDV and ACV at these chosen concentrations was found to be non-toxic for the cells since the lowest observed cell viability was around 78% (Figure 7)

Micrographs

At 4 d.p.i. of the infection study, a microscopic view of the wells was captured via a camera. In the negative control wells, uninfected cells were observed; uninfected cells were elongated and fiber-like in shape (Figure 8). A positive control well showed infected but untreated cells. Infected cells were round and enlarged in shape (Figure 9). The micrographs of infected wells pre-treated with different concentrations of antiviral drugs were each compared to the controls.

For the infection study, FOS at 5 µg/mL was combined with CDV at 1, 2.5, 5, and 7.5 µg/mL each. It should be noted that the cell viability at these concentrations were all greater than 80% (shaded region in Figure 6). Although some uninfected cells were observed in other combined drug concentrations (Appendix 6, 7, 8), the combination of CDV plus FOS at 7.5 and 5 µg/mL (Figure 10), respectively, was found to have the highest population of uninfected cells. A control of infected cells pre-treated only either with FOS (5 µg/mL) or CDV (7.5 µg/mL) was also observed. No uninfected cells were observed in well plates pre-treated with only 5 µg/mL FOS (Figure 11; all cells infected) while those pre-treated with 7.5 µg/mL CDV (Figure 12) contained some uninfected cells.

Acyclovir plus cidofovir was also used as an antiviral drug combination in this study. First, a control of infected cells pre-treated with only ACV at 10 µg/mL (Figure 13) or CDV at 4 µg/mL (Figure 14) was also observed. In both infected wells pre-treated only with ACV (10 µg/mL) or CDV (4 µg/mL), uninfected cells were observed. ACV (10 µg/mL) was combined with either 1, 2, 3, or 4 µg/mL of CDV. The aforementioned drug concentrations all had cell viabilities greater than 60% (shaded area in Figure 7). Although some healthy cells were observed in all the different combined drug concentrations (Appendix 9, Appendix 10, Appendix 11), the combination of ACV plus CDV at 10 and 4 µg/mL (Figure 15), respectively, was found to have the largest population of uninfected cells.

Plaque assays and combination index value

Plaque assays were carried out to quantify the virus present in each well plates. Plaque assays were only carried on the content of infected wells pre-treated with CDV (7.5 µg/mL), FOS (5 µg/mL), ACV (10 µg/mL), CDV (4 µg/mL), CDV (7.5 µg/mL) + ACV (10 µg/mL), and CDV + FOS (5 µg/mL).The number of plaques counted was then used to calculate drugs' concentration effect on the virus (Table 1) and the viral titer (Table 2). Based on effect of the drugs' concentration or dose, a combination index value plot was then generated in order to determine the effect of the drug combined together relative to that of the individual drugs. Combinations of CDV + FOS (Figure 16) and CDV + ACV (Figure 17) showed no effect and an additive effect against the virus (CI value = 1), respectively.

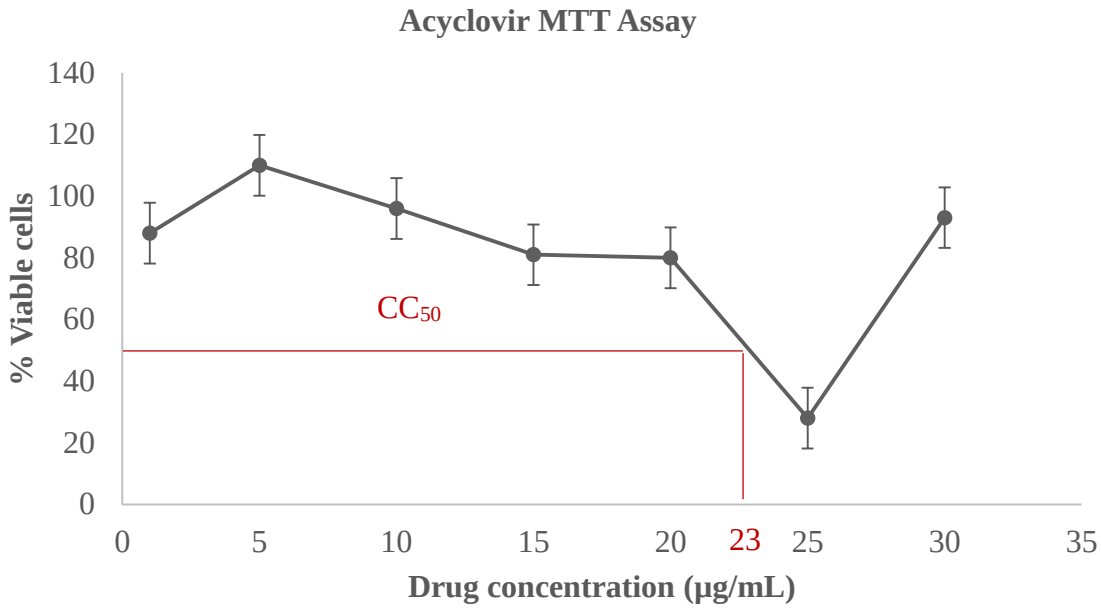


Figure 3: MTT assay of acyclovir. MTT assay was performed as explained in Materials and Methods. The percent of viable cells tends to decrease with increasing concentration of acyclovir. The red line indicates the CC_{50} of the drug, which was around 23 µg/mL. This assay was done in three replicates with error bars indicating mean ± 1S.E.

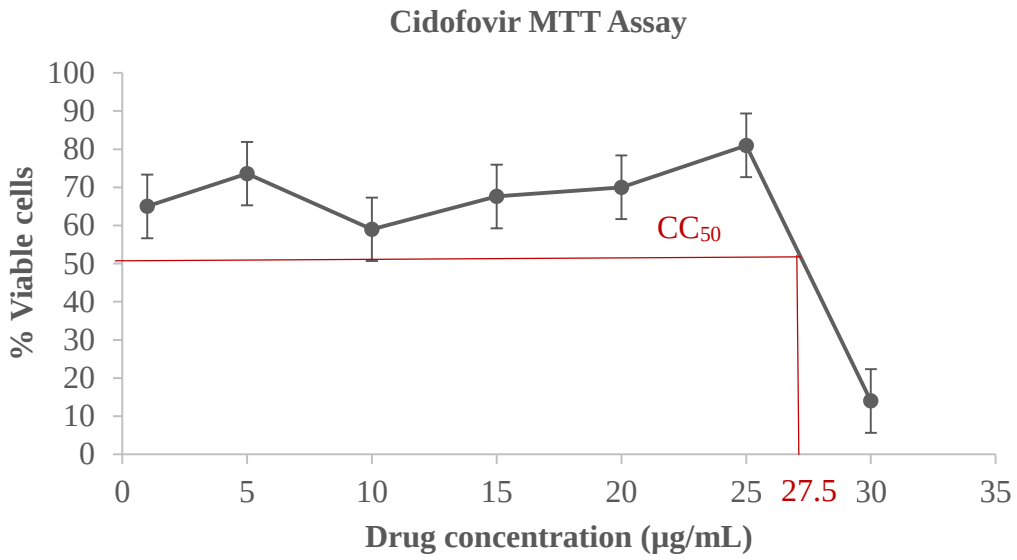


Figure 4: MTT assay of cidofovir. MTT assay was performed as explained in Materials and Methods. The percent of viable cells tends to decrease with increasing concentration of cidofovir. The red line indicates the CC_{50} of the drug, which was around 27.5 µg/mL. This assay was done in three replicates with error bars indicating mean ± 1S.E.

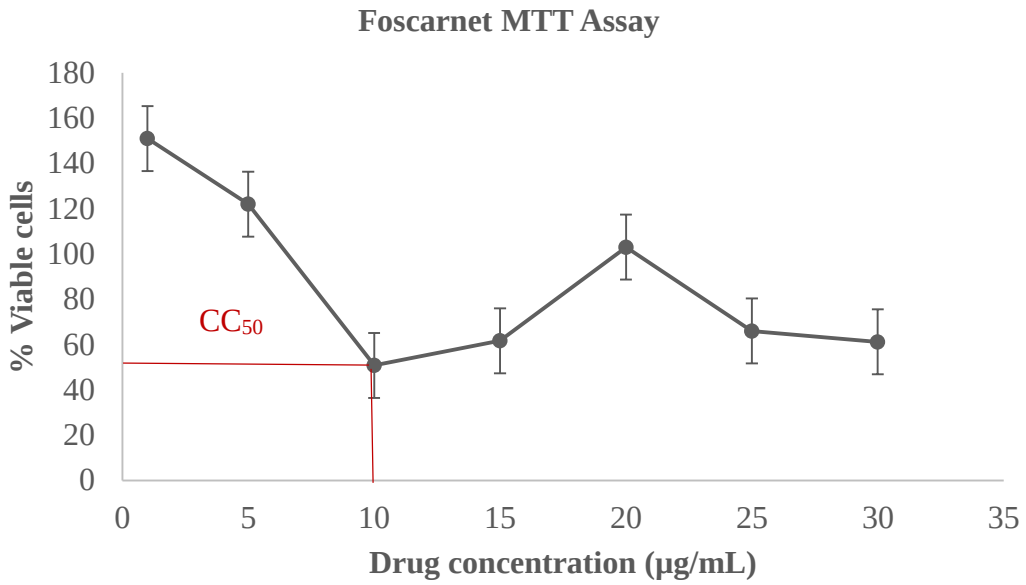


Figure 5: MTT assay of foscarnet. MTT assay was performed as explained in Materials and Methods. The percent of viable cells tends to decrease with increasing concentration of foscarnet. The red line indicates the CC_{50} of the drug, which was around 10 µg/mL. This assay was done in three replicates with error bars indicating mean ± 1S.E.

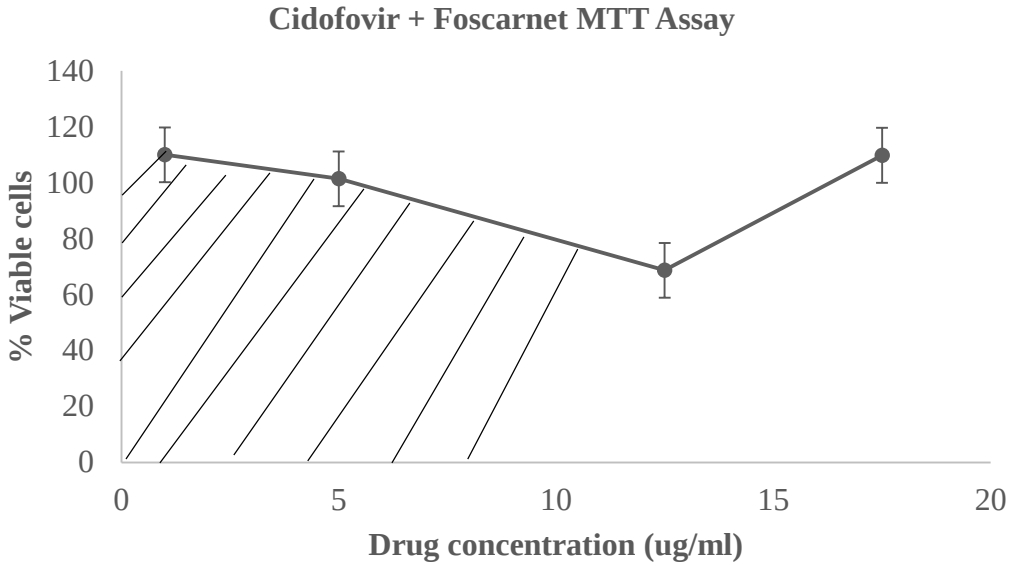


Figure 6: MTT assay of cidofovir + foscarnet. The drug concentrations used for this assay were all below the CC_{50} of individual CDV and FOS. Foscarnet was kept constant at a concentration of 5 µg/mL while CDV was varied at concentrations 1, 5, 12.5, and 17.5 µg/mL each. The shaded region represents the drug concentrations (foscarnet at 5 µg/mL combined with cidofovir at 1, 2.5, 5, and 7.5 µg/mL) used for the infection study (see below). It should be noted that the percent of viable cells in this region was far greater than 50%. This assay was done in three replicates with error bars indicating mean ± 1S.E.

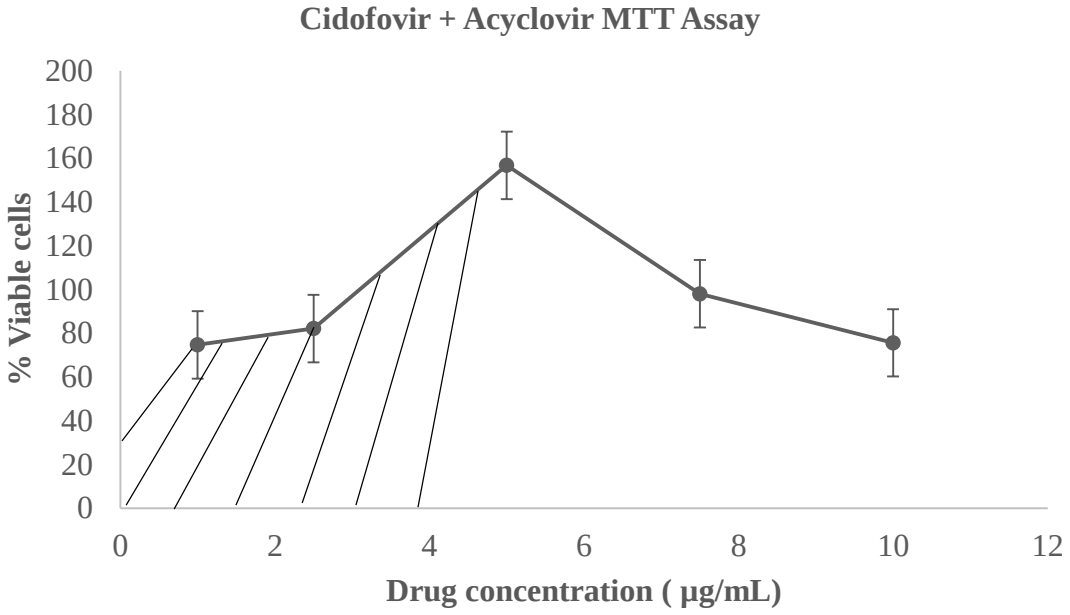


Figure 7: MTT assay of cidofovir + acyclovir. The drug concentrations used for this assay were all below the CC_{50} of individual cidofovir and acyclovir. Acyclovir was kept constant at a concentration of 10 µg/mL while cidofovir varied at concentrations of 1, 2.5, 5, 7.5, and 10 µg/mL each. The shaded region represents the drug concentrations (acyclovir at 10 µg/mL combined with cidofovir at 1, 2, 3, and 4 µg/mL) used for the infection study (see below). It should be noted that the percent of viable cells in this region was far greater than 50%. This assay was done in three replicates with error bars indicating mean ± 1S.E.

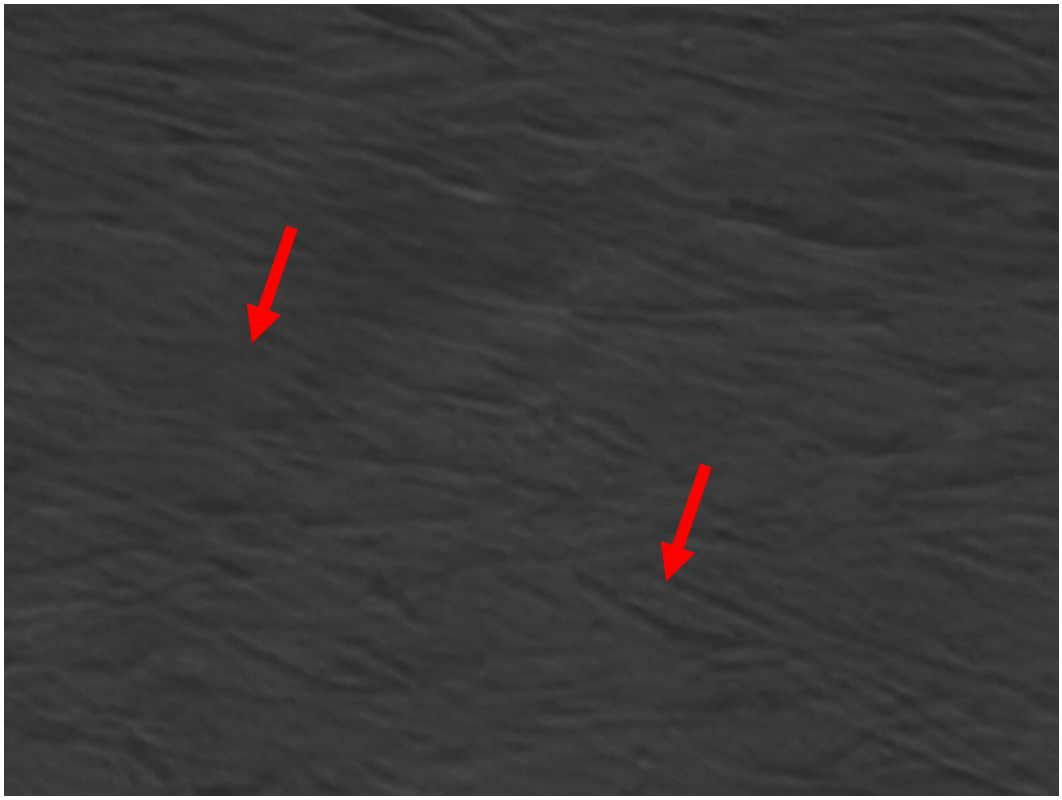


Figure 8: Micrograph of an uninfected well. Uninfected wells served as negative controls as they received no virus. Micrograph was taken at 4 d.p.i. The red arrows indicate uninfected cells, which were elongated and fiber-like.

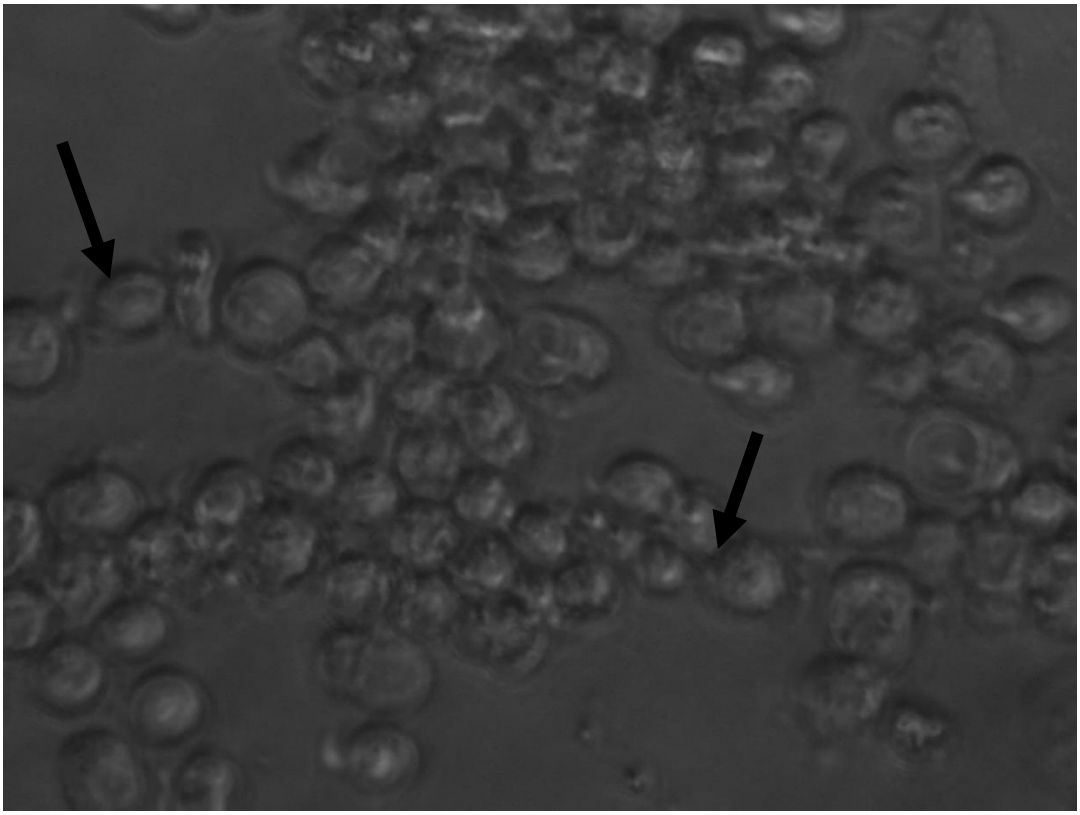


Figure 9: Micrograph of an EHV-1-infected well with no drug. EHV-1-infected wells with no antiviral drugs served as a positive control. Micrograph was taken at 4 d.p.i. Black arrows indicate infected cells, which were round and enlarged in shape.

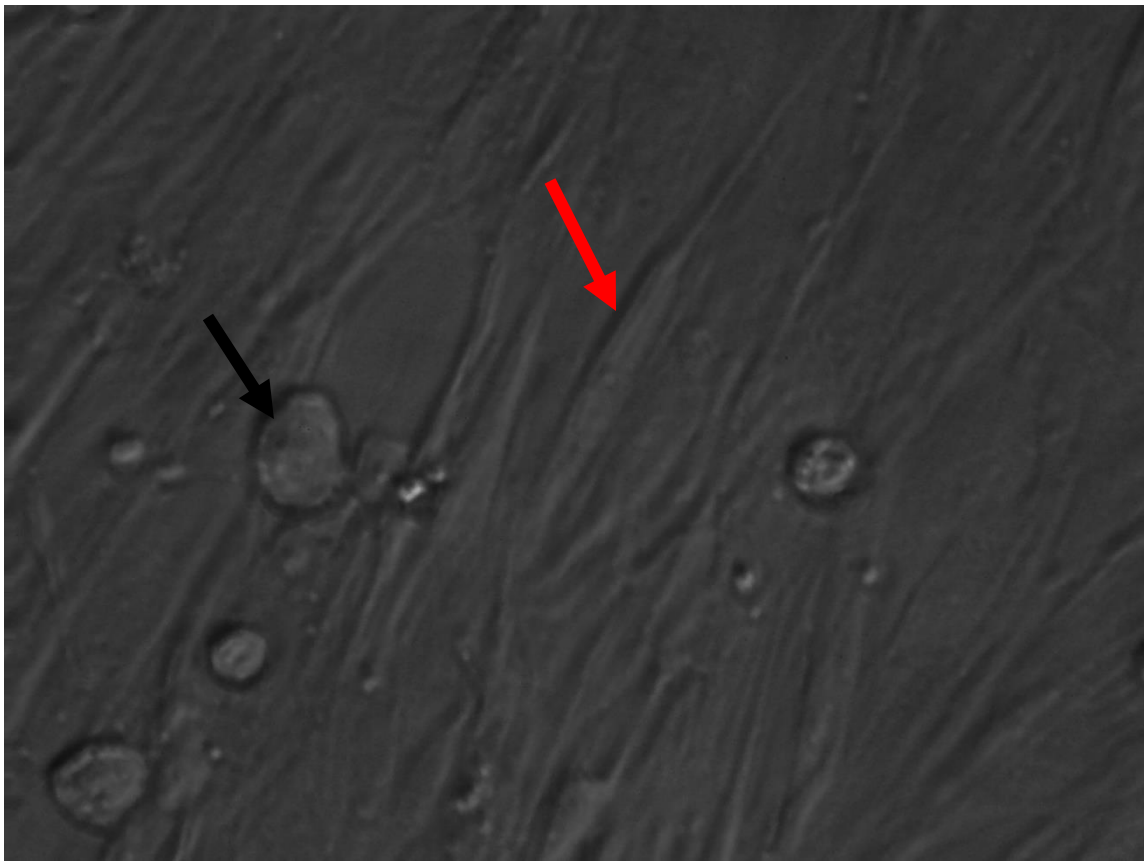


Figure 10: Micrograph of an infected well pre-treated with cidofovir (7.5 µg/mL) + foscarnet (5 µg/mL). Micrograph was taken at 4 d.p.i. Red arrow indicates uninfected cell, while black arrow indicates infected cell. The micrograph shows a high proportion of uninfected cells, indicating some protective effect of the drug combination against EHV-1 (T953).

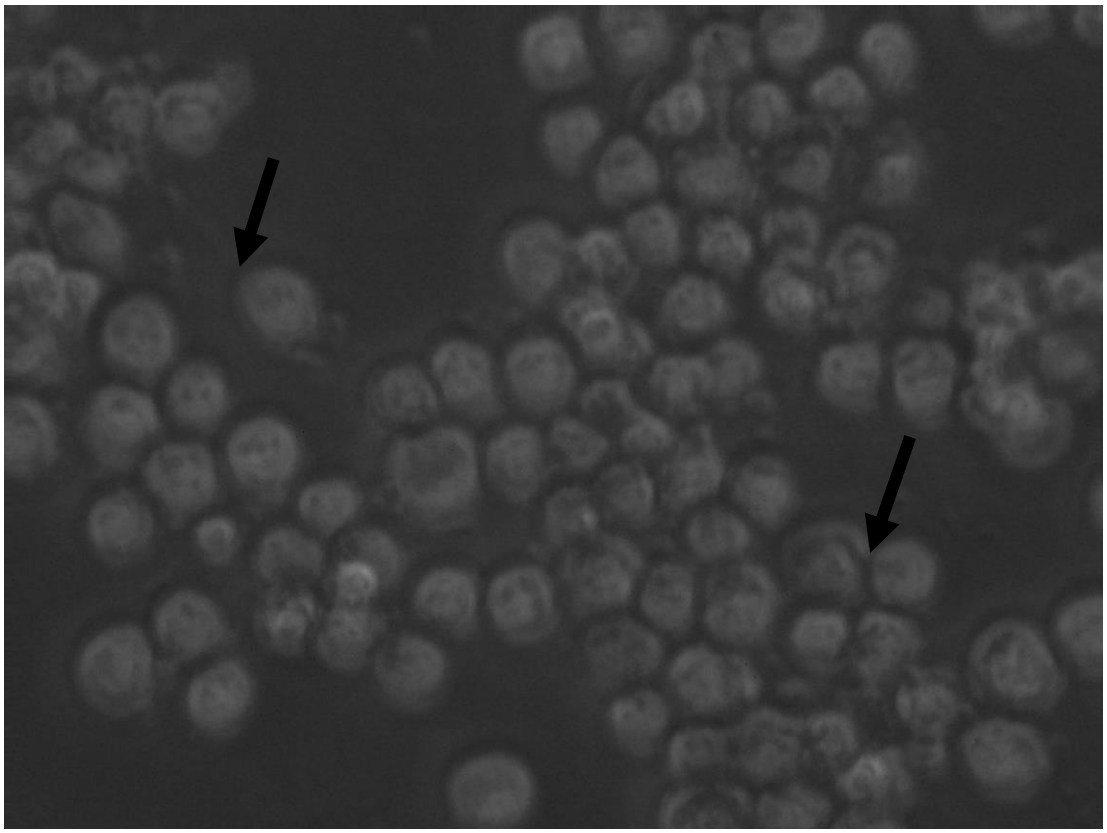


Figure 11: Micrograph of an infected well pre-treated with foscarnet (5 µg/mL). Micrograph was taken at 4 d.p.i. Black arrows indicate infected cells. The micrograph shows a high proportion of infected cells, indicating the ineffectiveness of foscarnet (5 µg/mL) in protecting the cells against EHV-1 (T953).

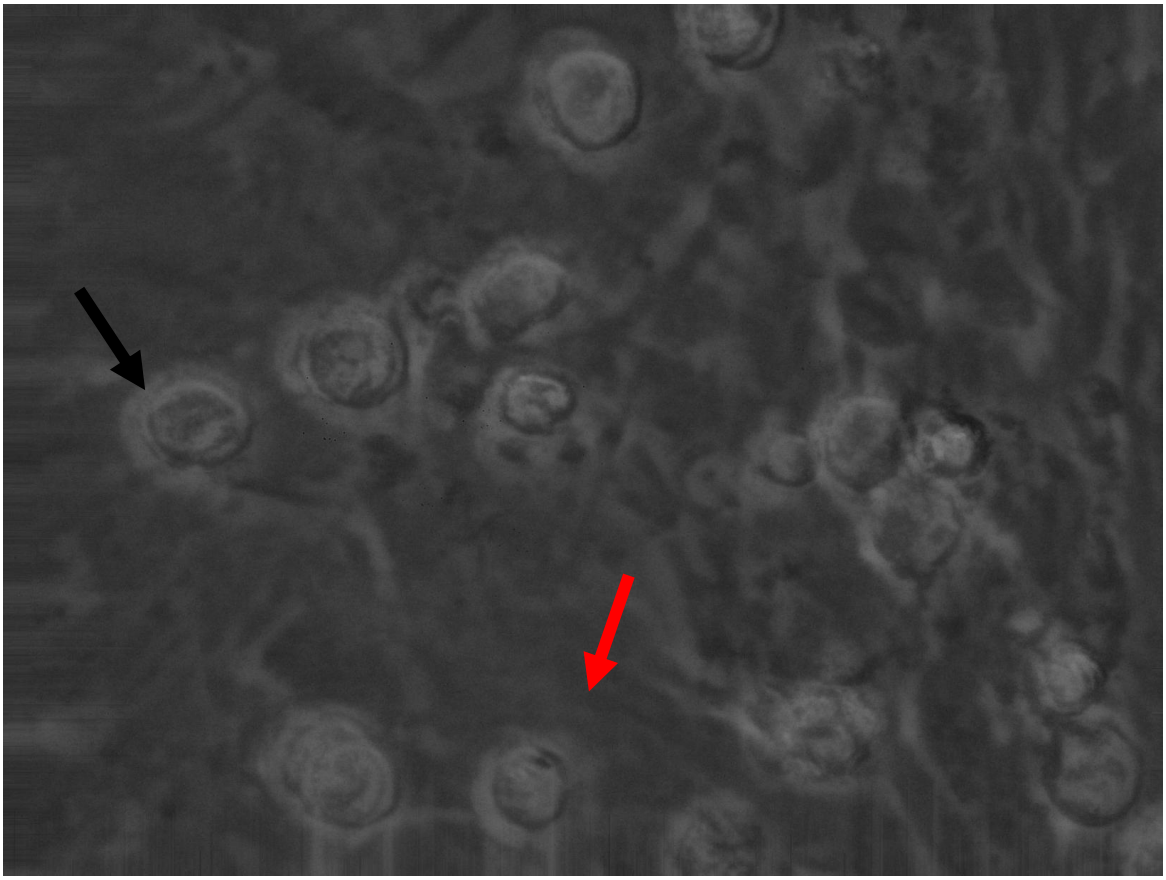


Figure 12: Micrograph of an infected well pre-treated with cidofovir (7.5 µg/mL). Micrograph was taken at 4 d.p.i. Red arrow indicates uninfected cell while black arrow indicates infected cell. The micrograph shows a high proportion of uninfected cells indicating some protective effect of cidofovir (7.5 µg/mL) against EHV-1 (T953).

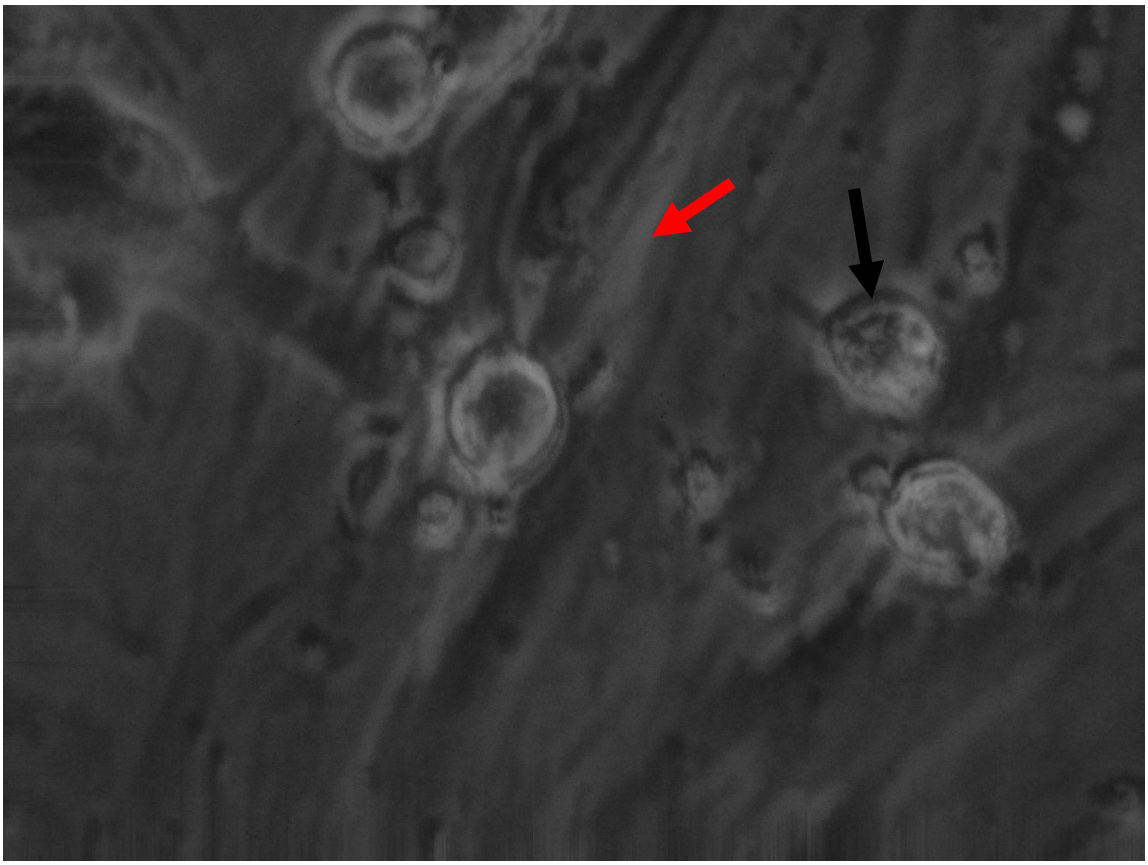


Figure 13: Micrograph of an infected well pre-treated with only acyclovir (10 µg/mL). Micrograph was taken at 4 d.p.i. Red arrow indicates uninfected cell, while black arrow indicates infected cell. The micrograph shows a high proportion of uninfected cells indicating some protective effect of acyclovir alone (10 µg/mL) against EHV-1 (T953).

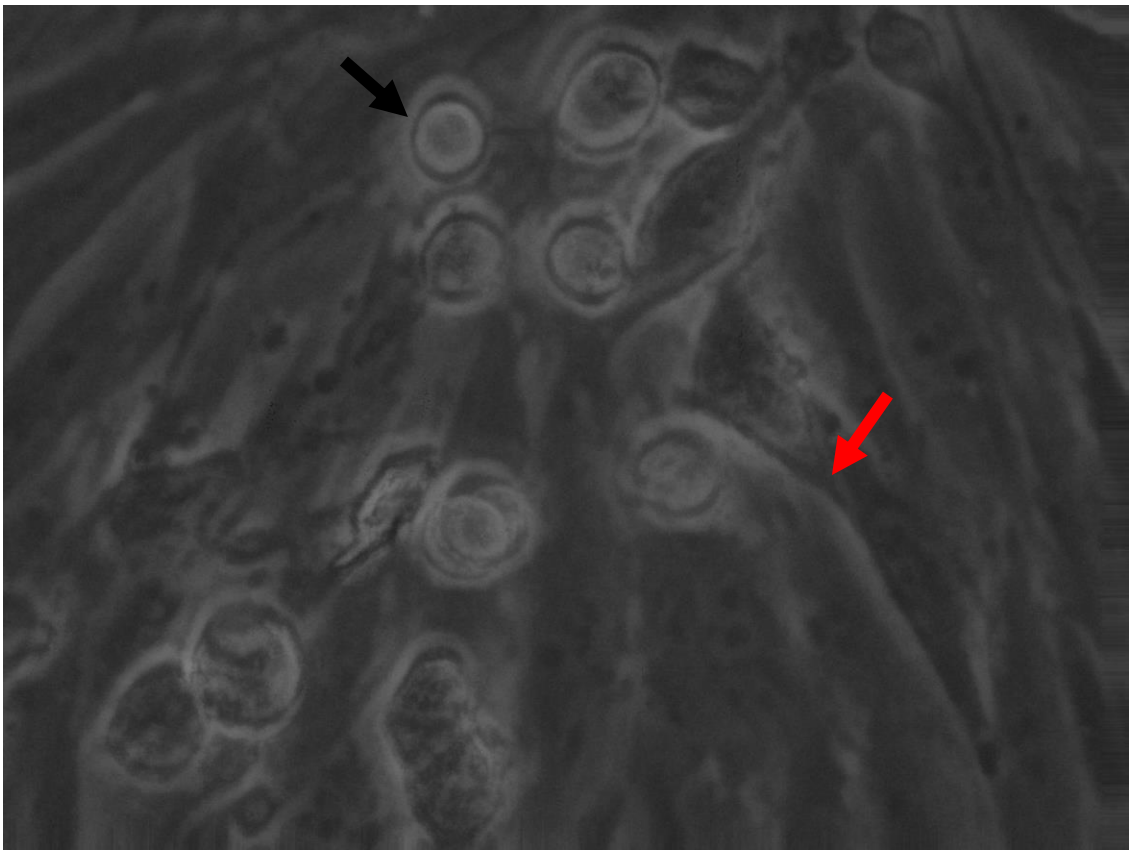


Figure 14: Micrograph of an infected well pre-treated with only cidofovir (4 µg/mL). Micrograph was taken at 4 d.p.i. Red arrow indicates uninfected cell, while black arrow indicates infected cell. The micrograph shows a high proportion of uninfected cells, indicating some protective effect of cidofovir (4 µg/mL) against EHV-1 (T953).

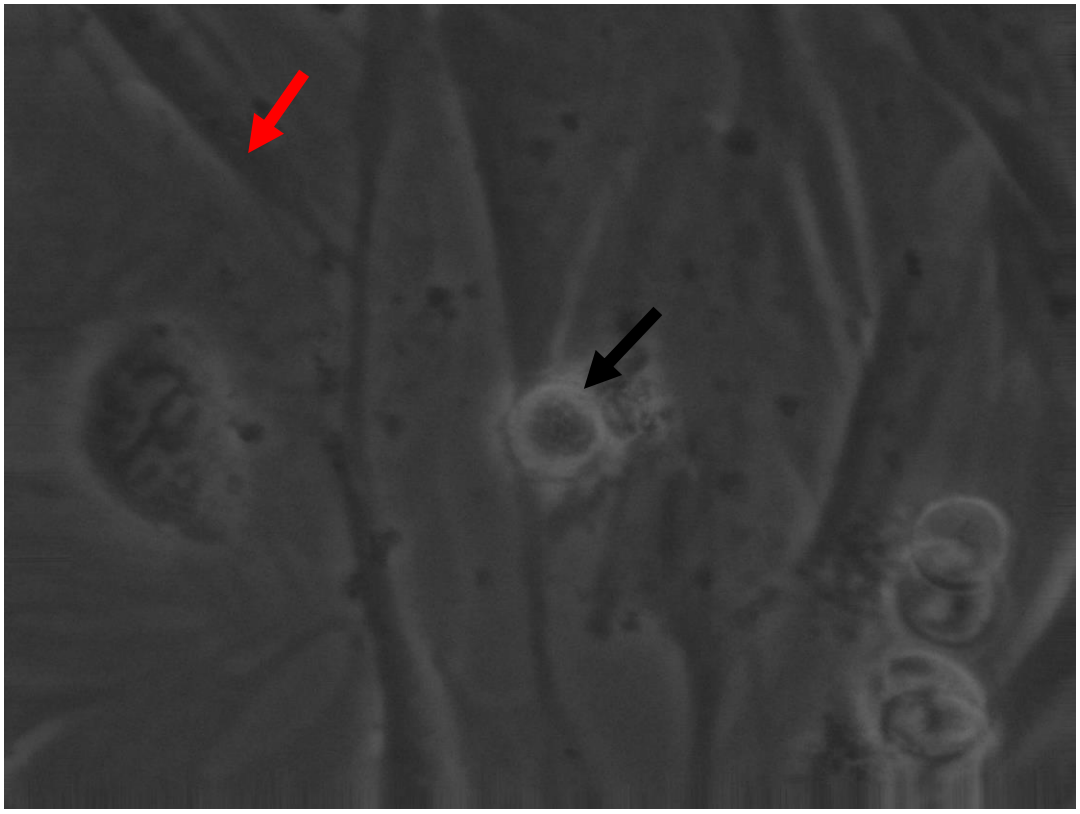


Figure 15: Micrograph of an infected well pre-treated with cidofovir (4 µg/mL) + acyclovir (10 µg/mL). Micrograph was taken at 4 d.p.i. Red arrow indicates uninfected cell, while black arrow indicates infected cell. The micrograph shows a high proportion of uninfected cells, indicating some protective effect of the drug combination against EHV-1 (T953).

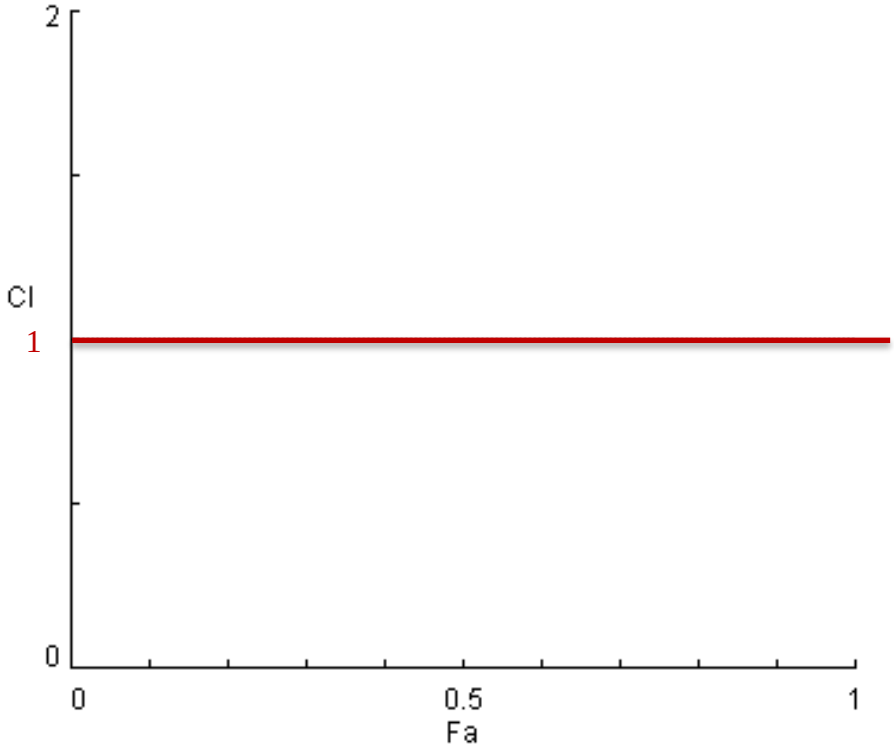


Figure 16: Combination index (CI) plot of cidofovir (7.5 µg/mL) + foscarnet (5 µg/mL). A combination index plot was generated as a function of fractional viral inhibition (Fa). A CI value less than 1, greater than 1 or equal to 1 indicates a synergistic, antagonistic or additive effect, respectively. The plot shows a CI value of 1, indicative of a no effect.

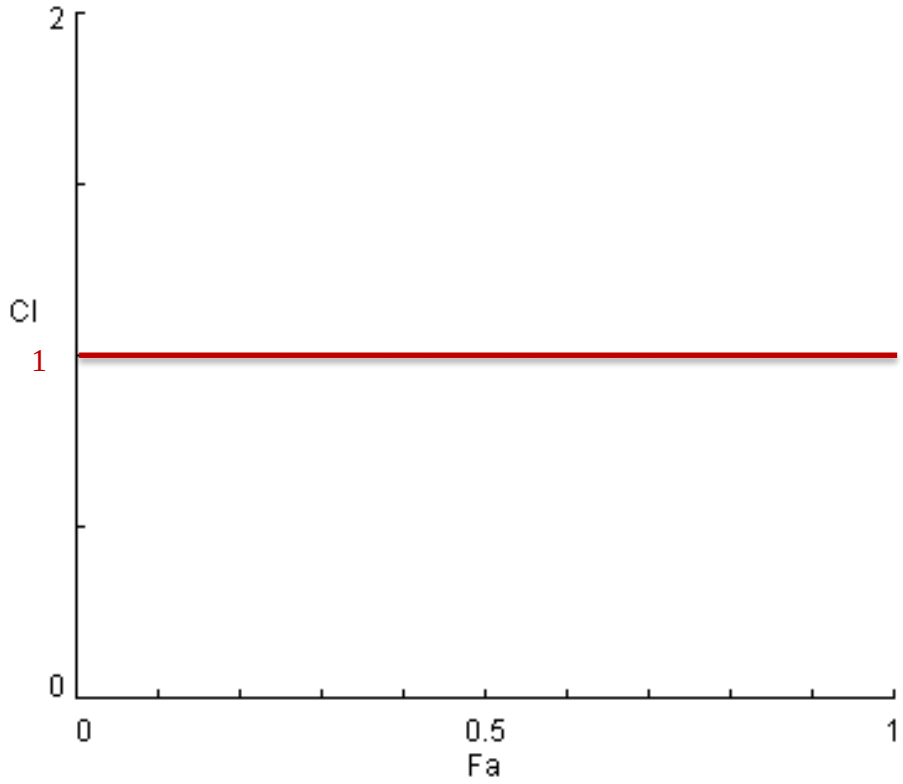


Figure 17: Combination index (CI) plot of cidofovir (4 µg/mL) + acyclovir (10 µg/mL). A combination index plot was generated as a function of fractional viral inhibition of (Fa). A CI value less than 1, greater than 1 or equal to 1 indicates a synergistic, antagonistic or additive effect, respectively. The plot shows a CI value of 1, indicative of an additive effect.

Table 1: Plaque assay counts and titers. The number of viruses present in infected wells pre-treated with antiviral drugs in combination or alone were determined via a plaque assay (see Materials and Methods). The plaque assays were done in duplicate for three independent replicates (R1, R2, and R3) of each treatment.

Antiviral drug dose (µg/mL)	**R1** **mean plaque number/viral titer (pfu/mL)**	**R2** **mean plaque number/viral titer (pfu/mL)**	**R3** **Mean plaque number/ viral titer (pfu/ml)**
Cidofovir (7.5)	150/750	144/720	156/780
Foscarnet (5)	264/1320	252/1260	276/1380
Cidofovir (7.5) + Foscarnet (5)	144/720	150/750	192/960
Acyclovir (10)	66/330	54/270	54/270
Cidofovir (4)	198/990	114/570	174/870
Acyclovir (10) + Cidofovir (4)	41/205	36/180	36/180
Negative control (No virus)	0	0	0
Positive control (infected but no drug)	280/1400	272/1360	240/1200

Table 2: Dose and effect of antiviral drugs alone or in combination. The effect of the various antiviral drug combinations were determined using the formula mentioned (see Materials and Methods). Note that the mean effect was calculated for 3 independent replicates plaqued in duplicates. These results were used to generate the Combination Index-Fa plot.

Antiviral Drug Dose (µg/mL)	**Mean Effect 1**	**Mean Effect 2**	**Mean Effect 3**
Cidofovir (7.5)	0.99975	0.99976	0.99974
Foscarnet (5)	0.99956	0.99958	0.99954
Cidofovir (7.5) + Foscarnet (5)	0.99976	0.99975	0.99968
Acyclovir (10)	0.99989	0.99991	0.99991
Cidofovir (4)	0.99967	0.99981	0.99971
Acyclovir (10) + Cidofovir (4)	0.999931	0.99994	0.99994

CHAPTER IV:
DISCUSSION

Non-toxic concentrations of antiviral drug combinations

The MTT assay demonstrated that the concentrations of both drug combinations (CDV + FOS, and CDV + ACV) were non-toxic for E. Derm cells. This was expected since these concentrations were chosen far below the CC_{50s} of their respective individual drugs. However, among the drugs used alone, FOS was the most toxic with a CC_{50} around 10 µg/mL, while CDV was the least toxic with a CC_{50} of 27.5 µg/mL. FOS is a pyrophosphate analog that inhibits DNA polymerase by mimicking the pyrophosphate product of DNA polymerization. Since FOS mimics a cellular compound (pyrophosphate), it was not surprising that FOS was the most toxic among ACV and CDV because it can also affect host DNA polymerases. CDV and ACV, on the other hand are nucleoside and nucleotide analogs, respectively, they are less likely to inhibit host enzymes. However, CDV (CC_{50} around 27.5 µg/mL) was found to be safer than ACV (CC_{50} around 23 µg/mL). Since ACV needs to be first activated by the viral thymidine kinase, it was expected to be safer than CDV when treating uninfected host cells. The fact that CDV was the least toxic indicates that some other host cell-specific activity, beyond its effect on host DNA polymerase, may be affecting its relative lack of toxicity in host cells. Similar results have been observed in another study (Garre *et al.*, 2007) where cidofovir was found to be less toxic than acyclovir. However, this previous study was done on equine embryonic lung cells using EHV-1 isolates different from the one used in this study. The discrepancy in these aforementioned cytotoxicity results of ACV and CDV may be explained by: cellular kinases having a lesser affinity for CDV as

compared to acyclovir; the cell's DNA polymerase being more sensitive to ACV triphosphate as compared to CDV triphosphate; or other unknown cellular enzymes which could speed up or aid in the first phosphorylation or use of ACV by the cell's DNA polymerase. Thus, it will be important to investigate the effect of equine kinases on ACV or CDV phosphorylation so as to determine the sensitivity, affinity or speed of these cellular kinases, and the way horses metabolize these drugs.

Also, the percentage of viable cells was sometimes found to be greater than 100%. This type of result was also observed in other previous studies (Garre *et al*., 2007). Viable cells actively metabolized are able to convert MTT into a purple-colored formazan product with an absorbance maximum near 570 nm, but the exact cellular mechanism of MTT reduction into formazan is not well understood; previous studies reported the involvement of reactions with NADH or similar mitochondrial reducing molecules that transfer electrons to MTT (Marshall *et al.*, 1995). When cells are non-viable, they lose this ability. While viabilities above 100% should not be possible, it should be noted that MTT reduction only reflects viable cell metabolism and not specific cell proliferation (Huyck *et al*., 2012). A previous study speculated the contribution of formazan crystals in harming cells by puncturing their membranes during exocytosis (Lu *et al*., 2012). Thus, it will be helpful in the future to use more sensitive assays such as ATP detection assays in the place of MTT assays (Riss *et al*., 2013).

Additive and no effect of antiviral drug combinations

In the case of CDV at 1, 2.5, 5, 7.5 µg/mL combined with FOS at 5 µg/mL, increasing CDV concentrations resulted in more uninfected cells. However, CDV at 7.5 µg/mL combined with FOS at 5 µg/mL was found to be the most potent in our hands,

since pre-treated infected wells showed the highest population of uninfected cells. Also, infected wells pre-treated with only FOS at 5 µg/mL exhibited only infected cells, while those pre-treated with only CDV at 7.5 µg/mL exhibited only some infected cells. A previous study reported FOS to be ineffective against different isolates of EHV-1 propagated in equine embryonic lung cells (Garre *et al*., 2007) .However, ours is the first report showing a visualization of the ineffectiveness of foscarnet on EHV-1 (T953) propagated in E. Derm cells. Since foscarnet was not found to be protective against the virus, any protective effect with CDV (7.5 µg/mL) + FOS (5 µg/mL) should be the result of a single effect of CDV. For that reason, plaque assays were carried out to determine the combination index value, which determines the nature of the drug combination effect against the virus, to ensure that FOS was not a contributing factor to the antiviral effect of CDV. Our data indicates that combining CDV (7.5 µg/mL) with FOS (5 µg/mL) leads to a no effect. This no effect shown with plaque assays confirms our visualization data whereby, the protective effect observed when combining CDV (7.5 µg/mL) + FOS (5 µg/mL) is as a result of CDV only. However, this result should be interpreted with caution since we only looked at one concentration of the drugs.

When CDV at 1, 2, 3, and 4 µg/mL was combined with ACV at 10 µg/mL, uninfected cells were observed with increasing concentrations of CDV. However, CDV at 4 µg/mL combined with ACV at 10 µg/mL was found to be the most potent because the wells exhibited the population of uninfected cells. It should be noted that the use of only CDV (4 µg/mL) or only acyclovir (10 µg/mL) also showed some protective effect against the virus. The nature of the effect (determined via plaque assays and combination

index analyses) was found to be additive. However, caution should be used in interpreting this result since we only looked at one concentration of the drugs.

Combining drugs is not a novel for the treatment of viral infections. Drug combinations have been used for the treatment of viral infections such as hepatitis C (Nakamoto *et al.*, 2015; Jacobsen and Sifontis, 2012) and HIV (Zhengtong *et al*., 2015). Combination therapies usually have the advantage of reducing doses of potentially toxic drugs, reducing the potential for the emergence of drug-resistant viruses, and increasing antiviral potency (Ellis *et al.*, 1989). For the treatment of hepatitis C virus (HCV), the use of antiviral drugs (daclatsavir and ledipasvir) combined with other direct-acting antiviral agents (ribavirin or peginterferon) were found to be efficient at tackling the emergence of drug-resistant HCV (Nakamoto *et al.*, 2014). The use of combination therapy have also been proven to be highly effective in the management of HIV infection (Volberding and Deeks, 2010). A combination therapy of HIV protease inhibitors, reverse transcriptase inhibitors, and/or intergrase inhibitor, commonly referred to as highly active antiretroviral therapy (HAART), is currently the most effective treatment against HIV (Zhengton *et al.*,2015). Because of HAART, AIDS-related mortality has dropped sharply, and AIDS is gradually becoming a controllable, chronic infection. (Zhengton *et al*., 2015). This combination therapy was also found to suppress virus replication to undetectable levels and to minimize the development of resistance (Este and Cihlar, 2010). Moreover, in the combination regimens, protease inhibitor based therapy was shown to have a lower level of resistance compared with non-nucleoside reverse-transcriptase inhibitor (NNRTI)-based therapy (Riddley *et al.*, 2008).

ACV is the first line of treatment choice of most herpes viruses' infections (HSV-1, HSV-2, VZV) and therefore seems to be an obvious choice for EHV-1 chemotherapy (Sellon and Long, 2007). However, resistance to ACV is a problem in immunocompromised human hosts since up to 7% of that population infected with herpes simplex virus type-1 (HSV-1) develop ACV resistance (Stranska *et al.*, 2005). Approximately 95% of HSV or VZV clinical isolates resistant to ACV is mainly due to the presence of TK-negative or TK-low producer phenotypes whereas a minority is due to presence of TK-altered and DNA polymerase-altered mutants (Roberts *et al.*, 1991; Malartre *et al*., 2012). Also, drug-resistant isolates selected by ACV therapy are generally cross-resistant to famciclovir, and the use of secondary therapies such as CDV and FOS are associated with significant toxicities (Prichard *et al.,* 2011). Thus, there is definitely a need for improved therapies for herpes infection in immunocompromised human hosts (Morfin and Thouvenot, 2003; Wilson *et al.*, 2009). Combination therapy has proven to be an effective way to treat ACV resistant HSV. A previous study reported a synergistic effect when combining topical acyclovir with another topical antiviral agent (A1110U) in the treatment of mice oro-facially infected with HSV-1. This synergistic chemotherapeutic efficacy was evident in infections caused by either ACV-susceptible or several ACV-resistant HSV-1 strains (Ellis *et al.,* 1989). Recently, it was found that combining CMX001 (a derivative of cidofovir) with standard ACV regimens may significantly increase the barriers to resistance and could be particularly useful in immunocompromised hosts (Prichard *et al.,* 2011). Therefore, drug combination therapies against equine herpes virus type-1 could be advantageous in decreasing the

emergence of drug resistant viruses or in reducing the toxic effect of antiviral drugs such as FOS.

To study the nature of the combined drug interaction, we used the CI-Fa plot by Chou (1984), a method widely used in pharmacology. However, this method is independent of the drug's mode of action (Berenbaum, 1981). Our combination index data showed that quantitative data may differ from qualitative data. This is because, the visualization of infected wells under the microscope, that are plates pre-treated with the drug combinations, may show a drug interaction to be synergistic because it appears that there are fewer infected cells than when either drug is used alone. However, one may actually be observing an additive effect that cannot be determined by visuals alone. Therefore, the quantitative effect of the plaque reduction must be used as the only true measure of potential synergy.

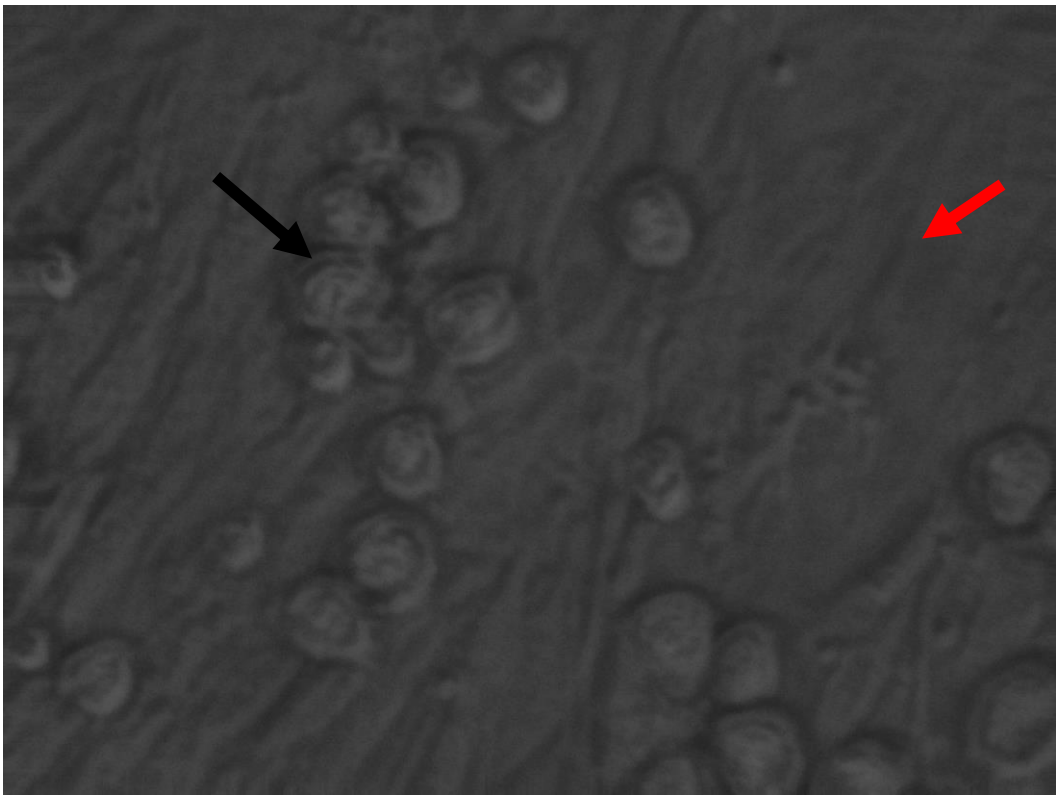
The nature of the effect when combining the drugs may look nil or additive now, but more data is definitely needed for detailed exploration of the CI values. Zhao *et al* (2005) reported that determining the effective drug concentration at 50%, 75%, and 90% (EC_{50}, EC_{75}, and EC_{90}, respectively) will give us a better and detailed exploration of CI values. Thus, in the future, we intent to look at different concentrations of the drug combinations, then use a dose-effect curve to generate a CI-Fa plot based on the drug's EC_{50}, EC_{75}, and EC_{90}. This will be helpful since we will be getting CI values based on the dose-effect of the drug across the whole study. Moreover, the CI-Fa plot generated in this study does not tell us anything about the combined action of the drugs. The plot just indicates the presence of an additive or no effect. Whether the mechanism of actions of the drug at the molecular level was additive or not still remains to be determined. Also, it

would be interesting in the future to use higher doses of CDV for the drug combinations. That is, the concentration of CDV in the combinations could be increased to up to 15 μg/mL and tested to see its effect against the virus.

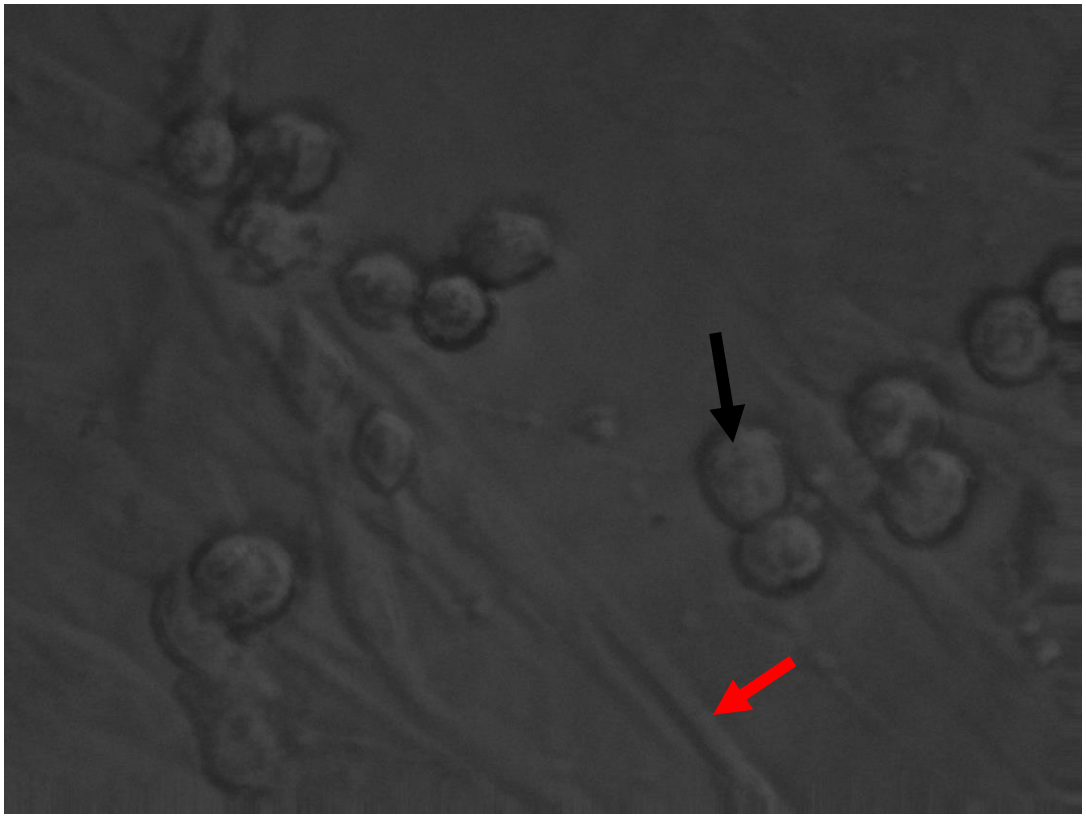
Conclusion

This study just showed preliminary data and more studies definitely need to be done in order to determine the effect of combined actions of the drugs relative to the dose-effect. Nonetheless, this is the first report indicating the additive effect of foscarnet plus cidofovir and acyclovir + cidofovir against EHV-1 (T953). Acquiring data from different doses of the drug combinations will enable us to look at the drug combination effect more specifically, and to generate a more detailed conclusion. This will also give us a glimpse as to what is happening specifically at the dosage-effect level of the entire cell study.

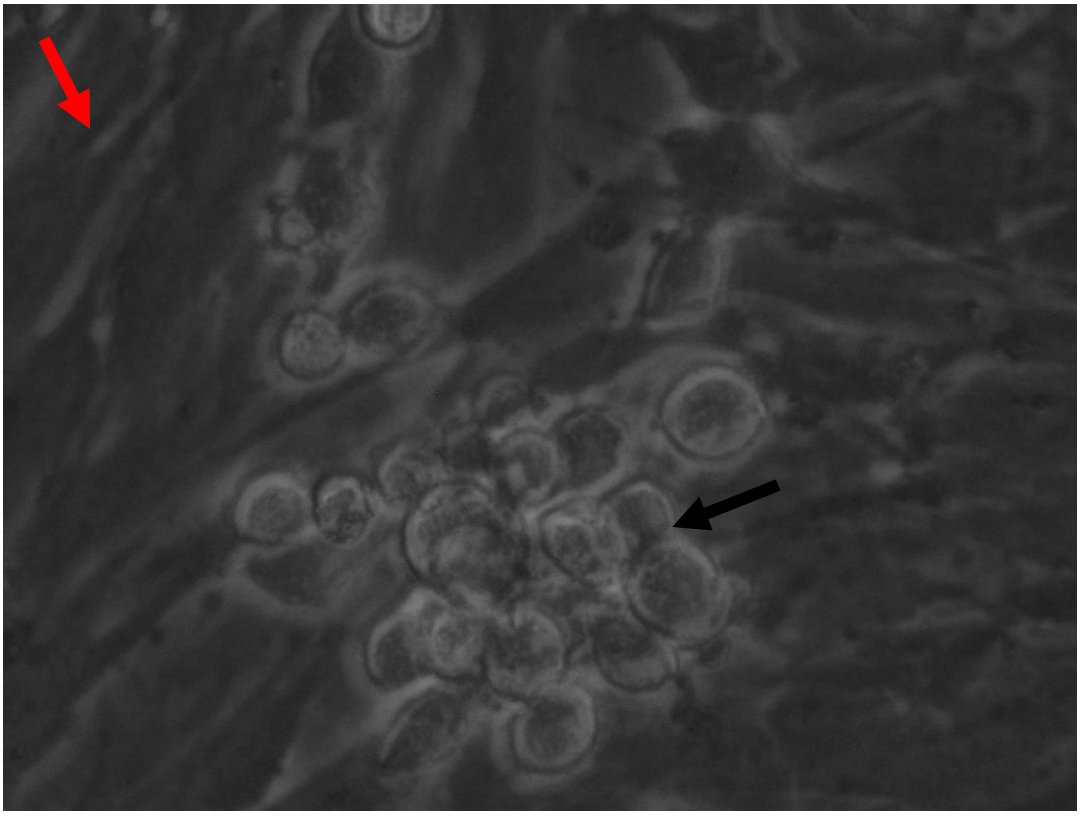
APPENDICES



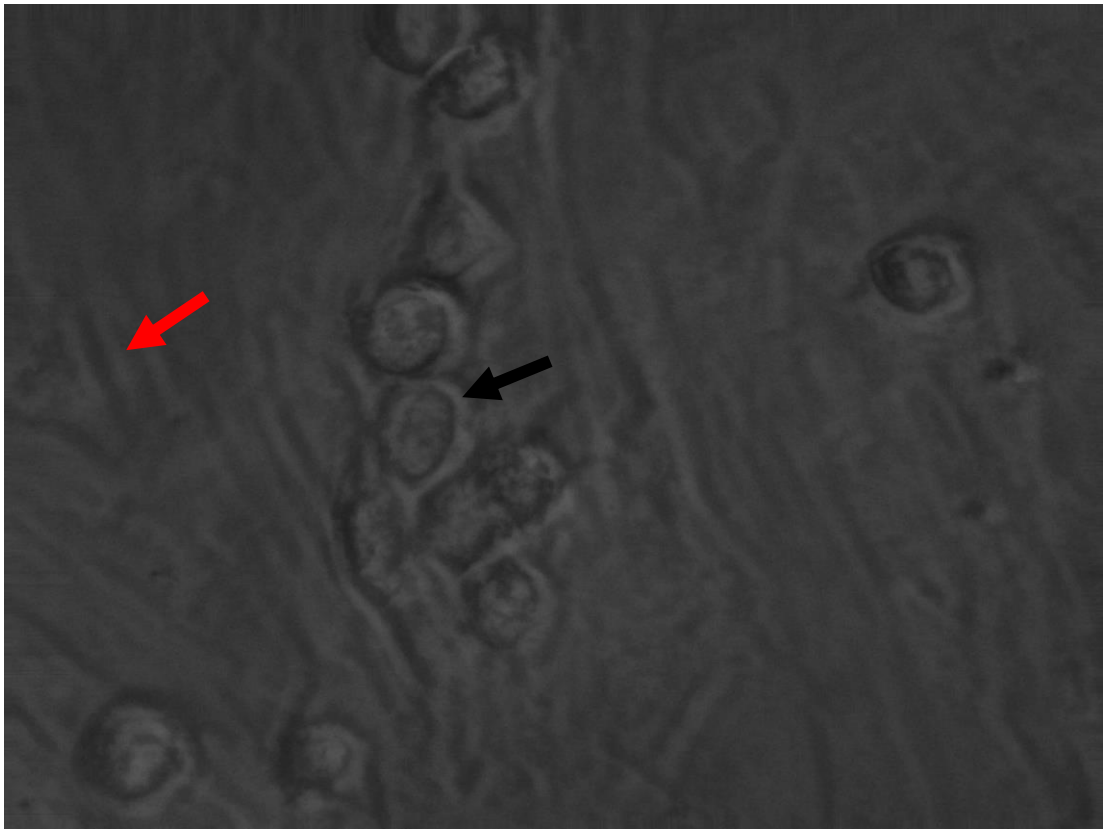
Appendix 1: Micrograph of an infected well pre-treated with CDV (1 µg/mL). Micrograph was taken at 4 d.p.i. Red arrow indicates uninfected cell, while black arrow indicates infected cell. The micrograph shows a high proportion of uninfected cells indicating some protective effect of CDV (1 µg/mL) against EHV-1 (T953).



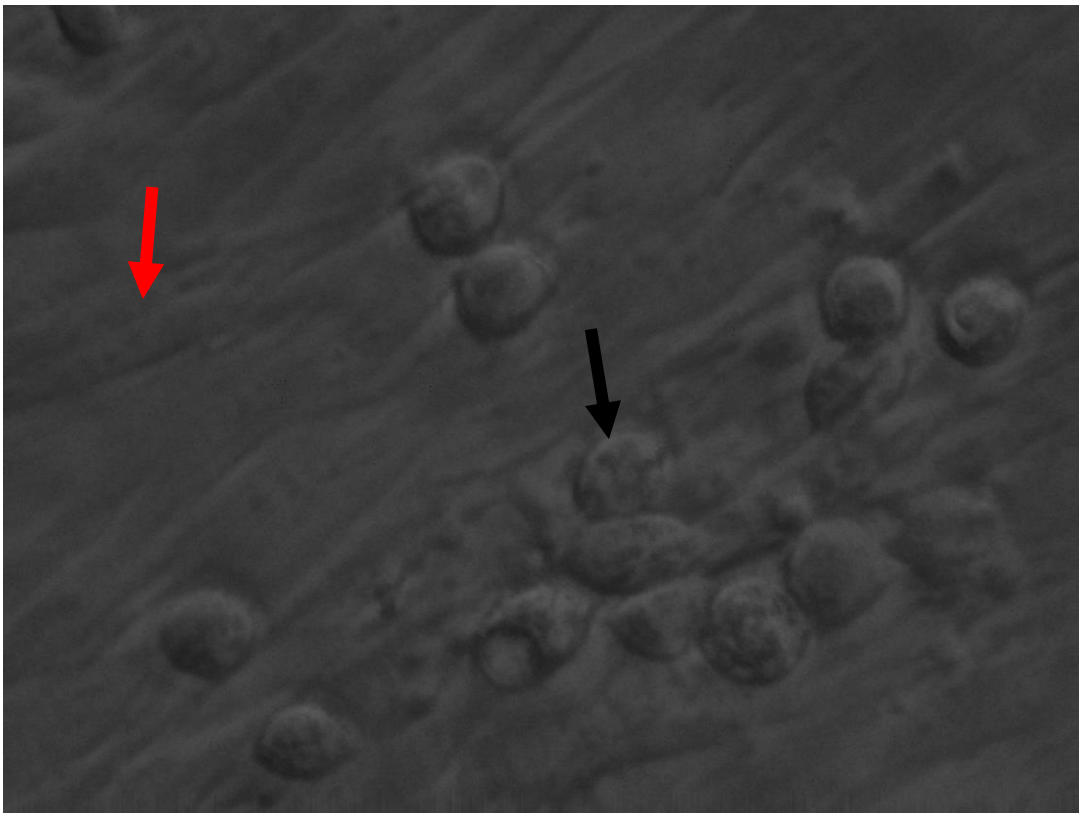
Appendix 2: Micrograph of an infected well pre-treated with CDV (2 µg/mL). Micrograph was taken at 4 d.p.i. Red arrow indicates uninfected cell, while black arrow indicates infected cell. The micrograph shows a high proportion of uninfected cells indicating some protective effect of cidofovir (2 µg/mL) against EHV-1 (T953).



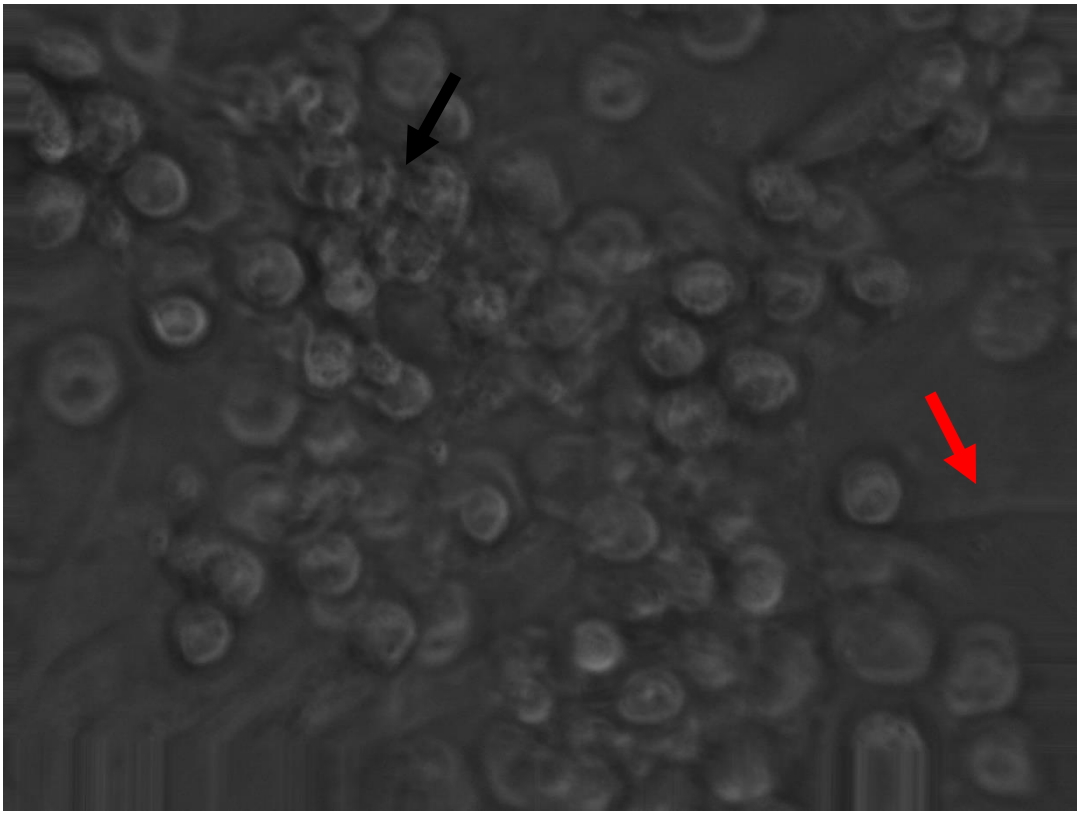
Appendix 3: Micrograph of an infected well pre-treated with CDV (2.5 µg/mL). Micrograph was taken at 4 d.p.i. Red arrow indicates uninfected cell, while black arrow indicates infected cell. The micrograph shows a high proportion of uninfected cells indicating some protective effect of CDV (2.5 µg/mL) against EHV-1 (T953).



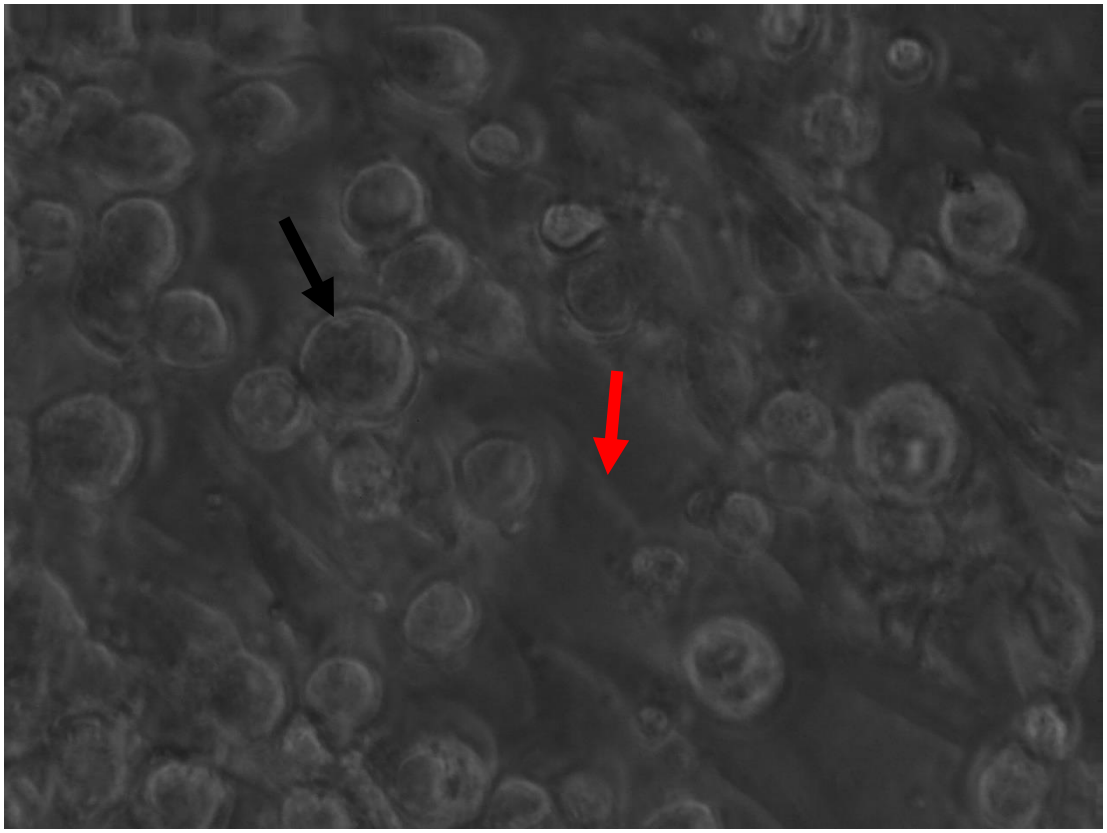
Appendix 4: Micrograph of an infected well pre-treated with CDV (3 µg/mL). Micrograph was taken at 4 d.p.i. Red arrow indicates uninfected cell, while black arrow indicates infected cell. The micrograph shows a high proportion of uninfected cells indicating some protective effect of CDV (3 µg/mL) against EHV-1 (T953).



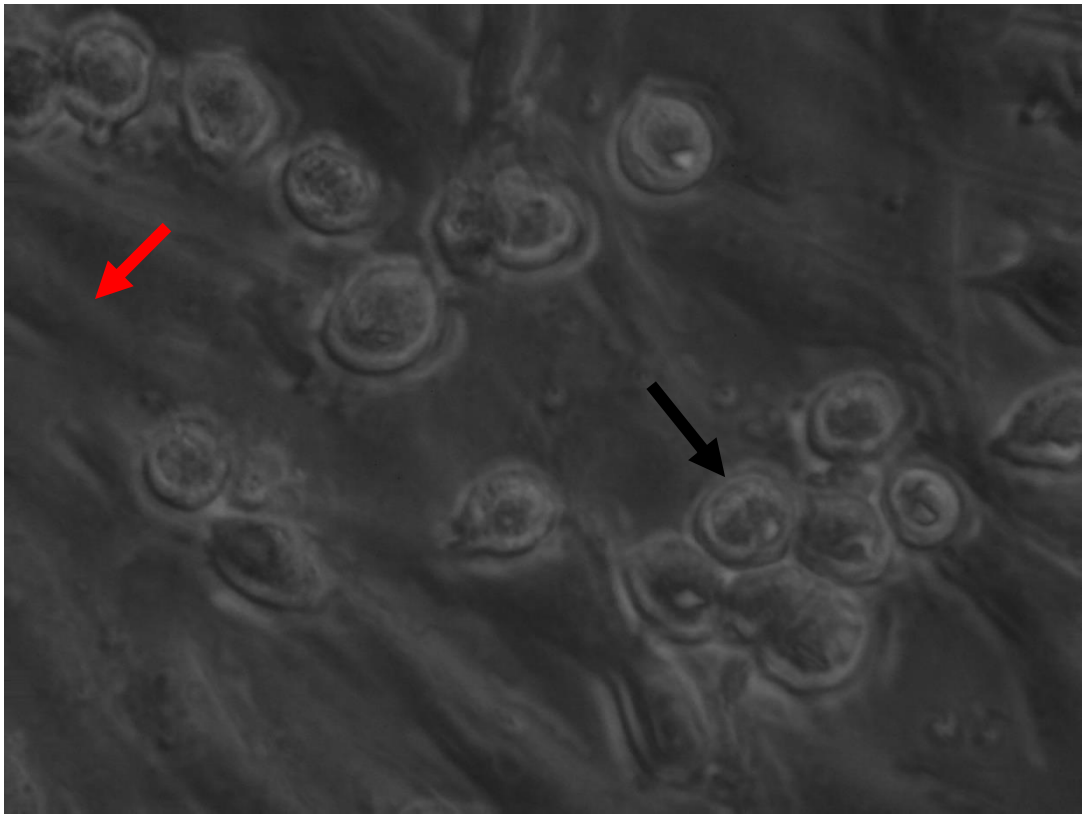
Appendix 5: Micrograph of an infected well pre-treated with CDV (5 µg/mL). Micrograph was taken at 4 d.p.i. Red arrow indicates uninfected cell, while black arrow indicates infected cell. The micrograph shows a high proportion of uninfected cells indicating some protective effect of cidofovir (5 µg/mL) against EHV-1 (T953).



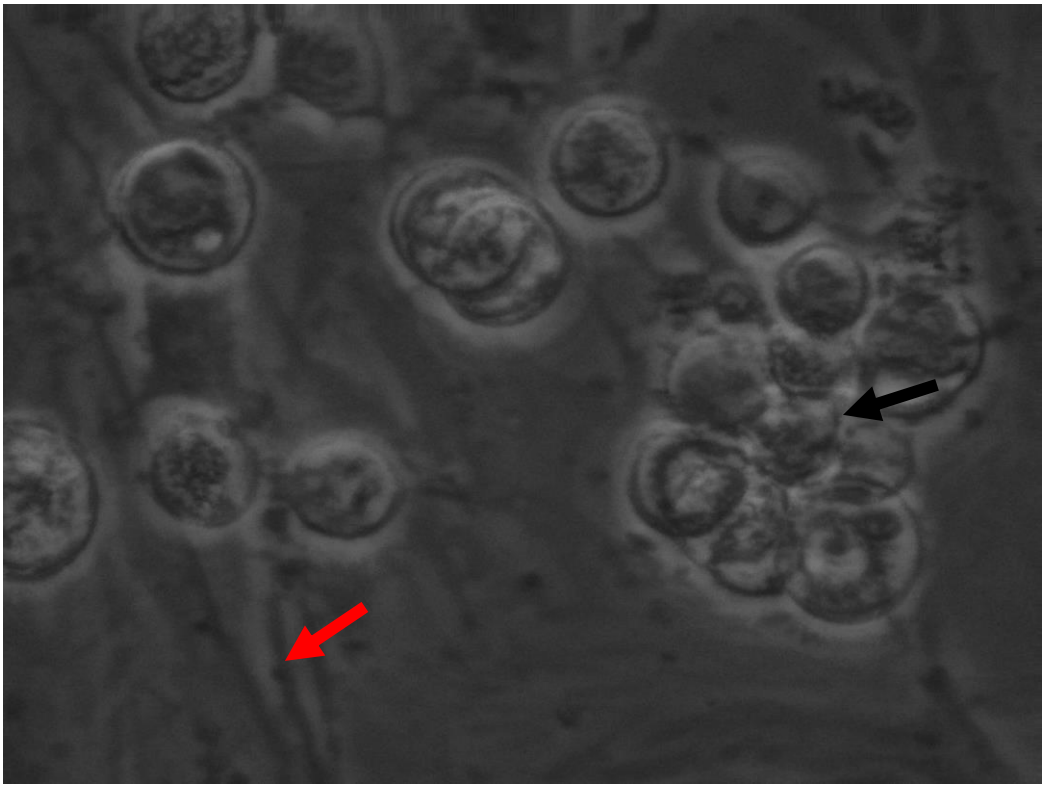
Appendix 6: Micrograph of an infected well pre-treated with CDV (1 µg/mL) + FOS (5 µg/mL). Micrograph was taken at 4 d.p.i. Red arrow indicates uninfected cell, while black arrow indicates infected cell. The micrograph shows a high proportion of infected cells indicating a non-protective effect of the drug combination against EHV-1 (T953).



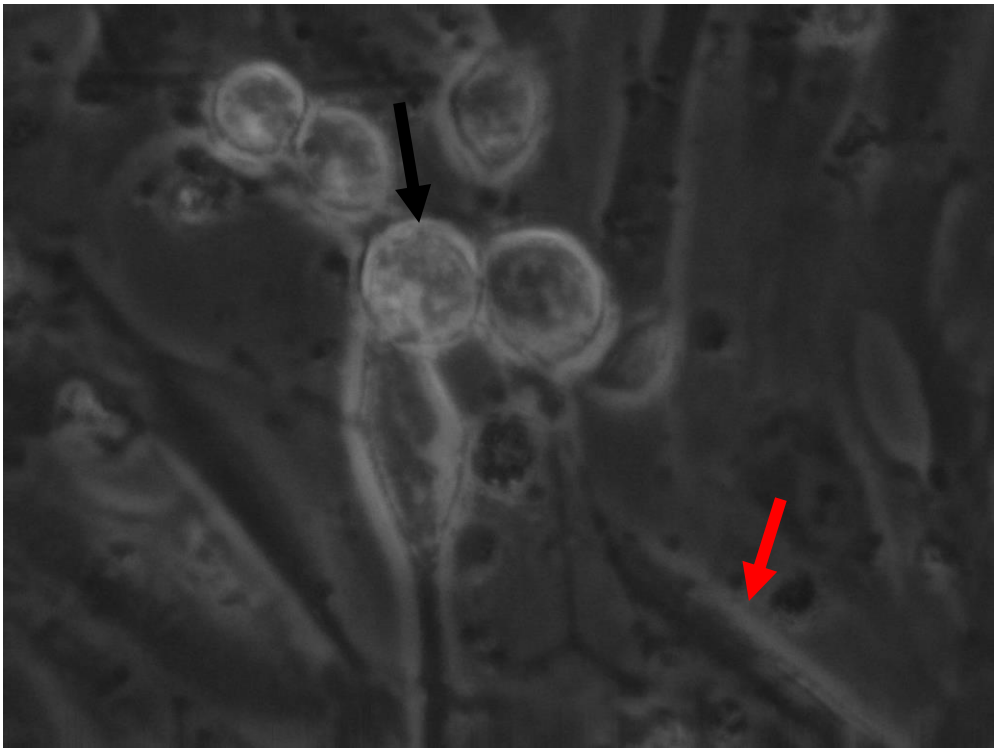
Appendix 7: Micrograph of an infected well pre-treated with CDV (2.5 µg/mL) + FOS (5 µg/mL). Micrograph was taken at 4 d.p.i. Red arrow indicates uninfected cell, while black arrow indicates infected cell. The micrograph shows a high proportion of infected cells indicating a non-protective effect of the drug combination against EHV-1 (T953).



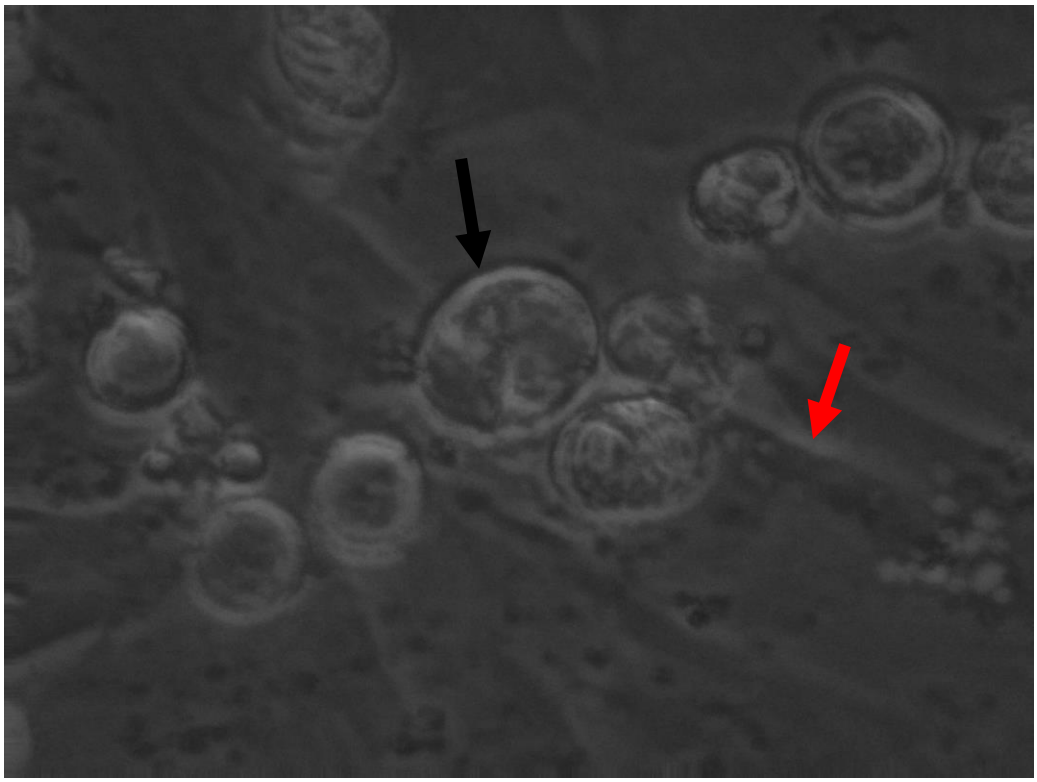
Appendix 8: Micrograph of an infected well pre-treated with CDV (5 µg/mL) + FOS (5 µg/mL). Micrograph was taken at 4 d.p.i. Red arrow indicates uninfected cell, while black arrow indicates infected cell. The micrograph shows a high proportion of uninfected cells indicating some protective effect of the drug combination against EHV-1 (T953).



Appendix 9: Micrograph of an infected well pre-treated with CDV (1 µg/mL) + ACV (10 µg/mL). Micrograph was taken at 4 d.p.i. Red arrow indicates uninfected cell, while black arrow indicates infected cell. The micrograph shows a high proportion of infected cells indicating a non-protective effect of the drug combination against EHV-1 (T953).



Appendix 10: Micrograph of an infected well pre-treated with CDV (2 µg/mL) + ACV (10 µg/mL). Micrograph was taken at 4 d.p.i. Red arrow indicates uninfected cell, while black arrow indicates infected cell. The micrograph shows a high proportion of heathy cells indicating some protective effect of the drug combination against EHV-1 (T953).



Appendix 11: Micrograph of an infected well pre-treated with CDV (3 µg/mL) + ACV (10 µg/mL). Micrograph was taken at 4 d.p.i. Red arrow indicates uninfected cell, while black arrow indicates infected cell. The micrograph shows a high proportion of heathy cells indicating some protective effect of the drug combination against EHV-1 (T953).

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Zhengtong, L. V., C. Yuan, and W. Yong. 2015. HIV protease inhibitors: a review of molecular selectivity and toxicity. *HIV/AIDS- Research and Palliative Care* **7**: 95-104.

CURRICULUM VITAE

Midrelle E. Nandjou

[redacted]

EDUCATION

August 2015 **Towson University**, Towson, MD
M.S. in Biological Sciences
GPA: 3.68/4.0

August 2011 **Buea University,** Buea, Cameroon
B.S. in Microbiology, Minor in Medical Laboratory Technology
GPA: 3.4/4.0

RESEARCH EXPERIENCE

08/2013-08/2015 **Towson University Biological Sciences Department**, Towson, MD
Graduate Researcher (Thesis): Investigates the synergistic effects of acyclovir, ganciclovir, and cidofovir with foscarnet against neuropathogenic equine herpes virus type-1 *in vitro*
Supervises and teaches laboratory techniques to students.

09/2010-06/2011 **University of Buea Microbiology Department,** Buea, Cameroon
Undergraduate Researcher: Characterized bacteria involved in the spoilage of Avocado pears and preventative techniques against these bacteria; wrote 32 page paper titled *Characterization of microorganisms involved in the spoilage of Avocado pear in South-West Cameroon*

06/2010-03/2012 **Biomedical Clinical Laboratory**, Douala, Cameroon
Medical Laboratory Technologist: Performed phlebotomy to collect blood for clinical analyses. Performed clinical biochemical and microbiological analyses on urine and vaginal secretions

TEACHING EXPERIENCE:

11/2013-present **Towson University Counseling Center**, Towson, MD
Alcohol and Drug Peer Educator: Taught three hour classes on a monthly basis to undergraduates with alcohol and drug issues; community outreach on the effects of alcohol, drug and substance abuse to the body

CONFERENCE PARTICIPATION

Nandjou M., Margulies B. Synergistic effects of acyclovir, ganciclovir, and cidofovir with foscarnet against equine herpes virus type-1 (EHV-1): Inhibitory drug concentrations for equine dermal fibroblast cells. *Host Pathogen Interactions in Biodefense and Emerging Infectious Diseases.* Manassas, VA. February 2015.

Nandjou.M., Margulies B. *S*ynergistic effects of acyclovir, ganciclovir, and cidorfovir with foscarnet against equine herpes virus type-1 *in vitro* : Inhibitory drug concentrations for equine dermal fibroblasts cells. *Virology Retreat.* College Park, MD, November 2014.

TALKS

Nandjou M. Synergistic effects of acyclovir, ganciclovir, and cidofovir with foscarnet against equine herpes virus type-1 (EHV-1) *in vitro. STEM majors*. Towson, MD. April 2015.

Nandjou M. Synergistic effects of acyclovir, ganciclovir, and cidofovir with foscarnet against equine herpes virus type-1 (EHV-1) *in vitro. Towson University Research Expo.* Towson, MD. April 2015.

RELEVANT COURSEWORK:

Graduate	**Undergraduate**
Virology	Cell Biology
Biotechnology	Biochemistry
Molecular Biology	Basic Pharmacology
Gene Regulation and Expression	Analytical Chemistry
Microscopy techniques and Staining	Genetics
Membrane Biology	Antimicrobial Chemotherapy
Data Analyses and Interpretations for Biologists (Biostatistics)	General Microbiology
	General Chemistry I & II with Lab
	Organic Chemistry I & II
	General Statistics

AWARDS AND HONORS

2014 **Towson University Graduate Student Association Research Grant**– $500 to fund thesis research

2009-2011 **University of Buea Scholarship** – Full Tuition Scholarship

TECHNICAL SKILLS:

Extensive Experience

Cloning, Mammalian Cell Culture, Bacterial Culture, Plaque Assay, Gel Electrophoresis, PCR, RT-PCR, Light Microscope, DNA/ RNA Miniprep, MTT assay Aseptic Technique, Differential/ Selective Media

Familiar Experience

ELISA, Phlebotomy, Antibiotic Susceptibility Testing, Tissue processing and staining, JMP (Statistical Software), Compusyn (Statistical software), CPR/AED Certified.